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ERYTHROPOIESIS WITH PARTICULAR REFERENCE TO ITS STUDY BY BIOPSY OF HUMAN BONE MARROW: A REVIEW

BY

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In this review we are attempting to present a picture of the morphology, physiology, and pathology of erythropoiesis in man. The ease with which the blood cells and their precursors may be examined provides an almost unique opportunity for their study and greatly adds to the charm and interest of haematology; and the results obtained thereby are probably significant for the growth of human tissues as a whole. Despite great progress in recent years, however, many gaps in knowledge and anomalies still exist, particularly in respect of the factors underlying the growth and differentiation of blood cells and the effects of pathological states upon them.

Because of the very great importance of technique, we are prefacing the main discussion with sections on the methods of bone marrow biopsy.

Methods of Bone Marrow Biopsy

The haemopoietic function of the red marrow has been realized since the description by Neumann in 1868 of the origin therein of the erythrocytes. The early studies on human material were carried out

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on post-mortem tissue, however, and it is only comparatively recently that biopsy of marrow has been widely employed and has thus rendered possible the examination of fresh material in stained smears. A big step forward in the technique of examination was made by Seyfarth (1923), who introduced a method of trephining the sternum by means of a small 3 mm. straight trephine. The preparation of histological sections from such material was admirably described by Custer (1933). However, although trephine biopsy provides excellent material for sections and smears, it has disadvantages; it is a minor surgical operation, which can be safely carried out only under aseptic conditions in the operating theatre, and which may be dangerous in the presence of a bleeding tendency or of granulocytopenia. In addition, the skin incision takes several days to heal, and the trephining can seldom be repeated. The introduction by Arinkin (1929) of the method of needle puncture biopsy of the sternum has, therefore, given a great impetus to the study of the marrow tissues during life. The relative values of the trephine technique and of needle biopsy are discussed by Dameshek and others (1937) and by Osgood and Seaman (1944). Stained smears of material aspirated after death are generally unsatisfactory, for the degenerating cells are easily broken up by the process of spreading (Rohr and Hafter, 1937; Leitner (Leitner and others, 1949a)).

Sternal puncture.—Arinkin's method of sternal puncture provides material which can not only be spread on slides, dried in the air, and stained like blood films, but which may also be examined supravitally by various optical techniques, and in addition provides marrow particles which may be sectioned or subjected to chemical microanalysis. The results obtained by Arinkin's technique in health and in many blood diseases have appeared in numerous papers and several monographs (for example, Segerdahl, 1935; Bodley Scott, 1939; Leitner, 1941; Rastelli, 1943).

A variety of needles exists. Some sort of adjustable guard is essential, and the needle designed by Salah (1934) is widely used in this country. In addition, needle-trephines have been designed; that of Türkel and Bethel (1943) is useful, yielding a small piece of cortical bone and underlying marrow. The trephine is passed through a hollow needle, and no skin incision is required.

In careful hands and using an aseptic technique sternal puncture is a safe procedure in the great majority of patients and may often be performed on out-patients. However, fatalities have resulted, mostly through complete transfixion of the sternum (*Lancet*, April 10, 1948), but this should not occur

if a guarded needle is employed. It should not be lightly undertaken in the presence of a bleeding tendency. Although, as a rule, little haemorrhage results in patients with a prolonged bleeding time, it should never be performed in haemophilia or in patients with similar coagulation defects.

The usual site for sternal puncture is the manubrium sterni or the first or second piece of the body of the bone; in our experience rather more cellular marrow with a smaller admixture of blood is obtained from the manubrium, although in this site it is rather difficult to be sure that the needle has entered the medullary cavity. The sternum may be repeatedly punctured; a different site should be selected for each puncture in order to avoid marrow possibly disorganized by haemorrhage resulting from previous punctures.

Other sites for needle biopsy; the tibia in childhood, the iliac crest, and vertebral spines.—In the youngest children, from birth to four or five years, the medial aspect of the head of the tibia may be punctured and active marrow withdrawn. This procedure is certainly less dangerous and less upsetting to the child than is sternal puncture. In older children the tibial cortical bone is usually too dense and the marrow within normally less active. Better samples are obtained by sternal puncture (Kato, 1937).

Recently the iliac crest (Rubinstein, 1948) and spinous processes of the vertebrae have been punctured and found in adults to yield good samples of marrow. Puncture of the spinous processes of the vertebrae is particularly convenient, as they lie superficially and the overlying tissues are not highly sensitive. The needle is passed vertically into the spines of the lumbar or lower thoracic vertebrae of the sitting or reclining patient (Loge, 1948). Rather more pressure is required than for sternal puncture. An added advantage of spinous process puncture is that the patient cannot see what is happening and that several attempts at puncture and aspiration can be made in the same anaesthetized area if necessary.

It is an advantage to have a choice of several sites, both in the same region, for example the sternum and vertebral spines, and between these areas, as repeatedly " dry " or non-cellular punctures are sometimes encountered.* In these cases, selection of a different site may either yield a cellular marrow or strengthen suspicion of a widespread pathological change affecting the marrow. The questions of different sites and of various forms of puncture needles are discussed at length by Rastelli (1943a).

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* Repeated failure to withdraw marrow is disconcerting. In our experience " dry " punctures or aspirates containing only blood or very few marrow cells have been due, in cases where we have been confident that the needle has penetrated the cortical bone, to marrow aplasia or hypoplasia, to marrow fibrosis, to secondary carcinomatosis, and sometimes to leukaemia, particularly of primitive cell types (see also page 4). Dameshek and others (1937) and Dameshek (1948) have had similar experiences. In these cases a surgical trephine operation may be required before the diagnosis can be made with certainty.

Although there is normally a considerable variation in the composition of cellular marrow withdrawn from adjacent or different sites, the general trend and type and maturity of haemopoiesis and the balance between erythropoietic and leucopoietic activity is similar (Pizzolato and Stasney, 1947). This essentially similar yet variable pattern is well shown when sections of aspirated material are studied (J. N. Davidson and others, 1948).

The Examination of Aspirated Bone Marrow

The preparation and staining of bone marrow films.—The volume of marrow which may be aspirated by puncture is limited, and the more material aspirated the greater the proportion of contaminating blood. Osgood and Seaman (1944) recommend that 1 ml. should be withdrawn as a routine, but we prefer not to take more than 0.2 ml.

Most, if not all, workers employ a Romanowsky dye for the routine staining of bone marrow films; many use a panoptic method, at a controlled pH (about 6.8) using May-Grunwald-Giemsa or Jenner-Giemsa stains. Both these techniques give excellent results, but in our experience a relatively long fixation with methanol (3–5 min.) is essential (Turnbull, 1948).* .

A well spread and well stained cellular film of marrow can be a delightful object, but the method of preparation of the film is as important as the staining technique. It is desirable to concentrate the marrow cells at the expense of the blood by which they are inevitably diluted, and various methods of concentration have been suggested. Davidson (1941) for instance expresses the contents of the aspiration syringe into a watch glass, picks out with forceps the fragments of marrow which adhere to the glass surface, and spreads them individually on slides. This technique provides marrow fragments with little contaminating blood and gives a better idea of the cellularity of the tissue than can be obtained by merely spreading drops of the aspirated material directly on to slides. Other authors (Limarzi, 1947) use more complicated methods (*vide infra*). We ourselves employ a very simple concentrating device which is generally satisfactory. A series of drops of syringe contents is delivered on to slides and most of the blood is then sucked off with a fine Pasteur pipette applied to one edge of the drop; the irregularly shaped

marrow fragments tend to adhere to the slide and are left behind. A film is then made of the marrow fragments and the remaining blood by means of a smooth-edged glass spreader of not more than 2 cm. in width; the marrow fragments are dragged behind the spreader and leave a trail of cells behind them. It is on these trails that differential counts should be done, commencing from the marrow fragment and working back towards the head of the film; in this way smaller numbers of cells from the peripheral blood become incorporated in the differential count.

Concentration of marrow by centrifugation.—Several workers have used centrifugation techniques in an attempt to assess the relative distribution of marrow cells, peripheral blood, and fat in aspirated material. Limarzi (1947) has devised a method for centrifuging heparinized aspirated marrow in a Wintrobe haematocrit tube. Yellow fat, red fat, plasma, marrow cells, and mature erythrocytes separate from above downwards. The marrow cell layer is used for preparing films and for differential cell counts. Limarzi (1947) claims that his method gives a relatively accurate indication of marrow hypo- or hyperplasia and that the amount of fat present can be gauged. He also claims that the separation of fat from the marrow cells facilitates their proper staining and that numbers of uniform preparations can be obtained from the sample. Berman and Axelrod (1947) have described a comprehensive method for treating aspirated marrow so as to obtain volumetric readings of the constituent fractions, as well as smears, imprints, and histological sections.

The data obtained by concentration techniques employing centrifugation are useful in group studies, but are less valuable in individual cases on account of the wide range of values encountered even in the normal.

Quantitative cell counts on aspirated marrow.—A number of figures for the cell content of aspirated normal marrow have been given in the literature (Osgood and Seaman, 1944; Vaughan and Brockmyre, 1947). The variation is extremely wide; this is hardly surprising in view of the uncontrollable factor of dilution with peripheral blood and the tendency of the marrow cells to adhere together in clumps of varying size. Perhaps the best method of enumeration is that of Isaacs (1937a), in which the particles of aspirated marrow are mixed with and broken up in normal serum. He attributed the dispersing action of serum to enzyme activity (Isaacs, 1930).

Tentative normal standards are given by Osgood and Seaman (1944). Fieschi, quoted by Rastelli (1943b), with whom we largely agree, states that no information is to be obtained from cell counts on the marrow that cannot be derived from assessment of simultaneously prepared films and sections. Clearly, the value of the quantitative method depends upon details of technique and is of greatest use in serial studies (see Stasney and Pizzolato, 1942).

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* Although Romanowsky staining is undoubtedly the best single staining method, there are simple supplementary techniques of value. By wet-fixing films in Susa fluid, pyronin-methyl green may be used to demonstrate nucleoli and cytoplasmic basophilia (White, 1947). Similarly Feulgen's staining reaction gives a good picture of the chromatin and chromosomes; light green yellowish S is a particularly suitable counter-stain. Phase-contrast techniques on unstained cells mounted in formalin have been used by Jones (1948) in the study of mitochondria.

The preparation of histological sections of aspirated bone marrow.—Although the operation of trephining the sternum provides excellent material for imprint preparations and for sections, the latter require decalcification which impairs staining, particularly by the Romanowsky dyes. It is, however, possible to prepare sections of the small fragments of marrow, free from bone, which may be obtained by simple aspiration through an ordinary puncture needle. These fragments of marrow, which Schleicher (1944a) has termed " marrow units," are naturally much smaller than the material obtained by surgical trephining, but they can be stained readily and they then provide a picture in miniature of the cellularity, proportion of fat spaces, and general architecture of the marrow. It is their small size, usually up to 1 mm. in diameter, which limits the value of the technique, for there is always some uncertainty as to how representative of the marrow the fragments are.

A number of techniques for sectioning these fragments have been described (for example, Davidson, 1941; Mertens, 1945; White and others, 1946; Cappell, 1947; Weisberger and Heinle, 1945), which differ in the details of handling the fragments and fixing and embedding. In none of these methods is it necessary to decalcify the tissue. These histological sections are quite easy to prepare; do they yield reliable and helpful information? The doubt as to whether the small fragments are really representative has already been referred to. In practice we find that although they are a useful adjunct to the examination of films, and reveal details of structure otherwise not easily appreciated, they do not often reveal more of diagnostic importance than do film preparations; indeed the latter are made from fragments similar to those sectioned. They certainly are less satisfactory for the study of subtle differences in haemopoiesis than are well stained films, particularly as the cytoplasmic structures are less well preserved. On the other hand they do indicate the relationships of the marrow cells one to another and to the parent reticulum cells and they show the proportion of the marrow occupied by fat and the relationships between sinusoids and the marrow cells. In addition it is easy to recognize cells such as phagocytic reticulum cells, which are difficult to identify in smears, and easily broken in the spreading of the film. Similarly megakaryocytes, which are only seen in smears in small numbers, are more prominent in sections, and occasionally a satisfactory and decisive view of tumour cells may be obtained.

Whilst relatively large fragments of marrow are obtained by simple aspiration on most occasions, we have sometimes been unsuccessful just when it seemed most important to obtain material. In these patients, mostly suffering from marrow aplasia or hypoplasia, marrow fibrosis (" myelosclerosis "), secondary carcinomatosis, or aleukaemic leukaemia, the films are similarly uninformative. In myelosclerosis and in carcinomatous infiltration the tissue is presumably too firmly organized, with a thickened reticulum and perhaps an increase in collagen also, to be disrupted by suction. In the leukaemias and in " aplastic " anaemia it is more difficult to understand why aspiration should fail; sometimes it is difficult even to obtain blood. Possibly the ease with which material is withdrawn depends not only on the texture of the marrow, in particular the volume of developing marrow cells compared with the basic reticular structure, but also on its blood supply. If the blood circulating through the bone marrow is restricted, as it might be, due to proliferation of leukaemic cells or perhaps to marrow aplasia, difficulty in aspiration might result; for it is clear that, when one is aspirating from a small enclosed area, blood must enter to replace the material withdrawn. In practice, if the operator fails to obtain marrow from the sternum, it is advisable to try another site such as the lumbar spine. If aspiration of the spine is unsatisfactory, trephining through an introducer needle (Türkel and Bethel, 1943) or surgical trephining of the sternum may be needed.

In Plate I are illustrated photomicrographs of sections of fragments of marrow from patients suffering from a variety of blood disorders. The specimens were obtained by aspiration of the manubrium of the sternum and prepared by the method of White and others (1946).

The Extent of the Normal Marrow at Different Ages

As the normal distribution of the bone marrow has a bearing on the selection of sites for biopsy, this question will be briefly considered. At birth, active red marrow is found throughout all the ossification centres in the skeleton, and puncture of the tibia medial to the tubercle will yield active marrow in young children. The regression of active marrow from the long bones in later childhood and adolescence and the partial regression in the flat bones is well illustrated by Custer and Ahlfeldt (1932). The active red marrow is replaced by fat in which the potentially haemopoietic reticulum cells normally lie dormant. Under abnormal conditions, such as in many anaemias and in the leukaemias, these reticulum cells once more become active and form haemopoietic stem cells; the fat cells may almost completely disappear. There does not seem to be any striking difference between the extent of the marrow once the growth period is completed. There is no significant difference in distribution and cellularity between the marrows of elderly subjects and normal adults (Reich and others, 1944; Leitner and others, 1949b).

The cause of the apparent increase in volume of the marrow in childhood is uncertain; it may in fact be more apparent than real. It is not certain that the volume of marrow related to the child's total blood-cell volume, which it constantly maintains, is any greater than the marrow-total-blood-cell volume relationship in the adult. A possible cause of a hyperplastic marrow would be that the erythrocytes (and leucocytes) in childhood had a shorter life than in the adult. Mollison's (1948)

observations on the survival of the blood of newborn infants in other normal infants suggests, however, that postnatal erythrocytes are likely to survive for a normal length of time. Moreover, the total granulocyte count in childhood and adolescence is also very similar to that of the adult, which suggests that the demand on the marrow for granulocytes is similar throughout life and is not likely to be a cause of marrow hyperplasia.

Normal erythropoiesis : site of development.—The exact site of erythropoiesis in the normal marrow and the problems connected with the haemopoietic potencies of the more primitive marrow cells have been a source of controversy for many years. Although it is not our intention in this paper to review in detail the many theories of haemopoiesis that have been suggested, a limited treatment is required. A cardinal point in the polyphyletic theories of Sabin, Cunningham, and Doan and co-workers has been that, whereas granulocytes are formed extravascularly, the erythrocytes arise intravascularly from the lining endothelium of intersinusoidal capillaries (Doan, 1923; Doan and others, 1925). Their evidence was based partly upon the study of regeneration occurring in the hypoplastic fatty marrow of pigeons previously starved. However, granulocytes and erythrocytes do not seem to develop in this way in man and in other mammals, and an extravascular origin for both has been claimed by the majority of workers (Bunting, 1906; Maximov, 1910; Drinker and others, 1922; Bloom, 1938a). More recently Hamre (1947) has described the extravascular formation of erythrocytes and granulocytes in rats and the subsequent migration of the more mature cells across the intact sinusoidal endothelium.

Study of our own sectioned material has convinced us of the correctness of the conception of extravascular haemopoiesis in man. The more primitive haemopoietic cells in both normal and abnormal marrows always seem to be in the stroma of the marrow and outside the marrow sinusoids and capillaries.* In the sinusoids, however, there may be seen a few partly mature forms, such as polychromatic normoblasts and metamyelocytes, in addition to mature erythrocytes and leucocytes.

If it is admitted that haemopoiesis is extravascular, the problem of the migration of the developing blood cells from the marrow stroma into the blood stream remains to be solved. In the case of granulocytes, with their known capacity for amoeboid movement, the migration of the more mature forms across an endothelial barrier presents little difficulty and is generally admitted to occur, but the movement of the non-motile normoblasts is more difficult to understand. In Drinker and others' (1922) view it was the pressure of expanding collections of maturing normoblasts that forced the latter through the sinusoidal endothelium. Maximov (1910) and Key (1921) postulated temporary deficiencies in the continuous endothelium but did not produce conclusive evidence of this. Hamre (1947) has failed to demonstrate in rats any such breaches in continuity. He observed the passage of both granulocytes and erythrocytes through the sinusoidal walls, and considered that the cells passed between the margins of the lining endothelial cells and that pressure from the growing mass of cells in the stroma might well help them to do so. Our evidence from human material points in the same direction; in hyperplastic marrows such as are seen in Addisonian pernicious anaemia we have observed groups of cells bulging into the lumina of the sinusoids. Isaacs (1930) has called attention to the mutual adhesiveness of the more primitive marrow cells. As the cells mature they become free from one another through liquefaction of the intercellular material and come to lie in " lake-like " spaces. This separation is a necessary preliminary to a cell's entry into the vascular sinusoids proper. The relationship between erythrocyte or normoblast migration through the sinusoidal walls and the formation of poikilocytes will be considered in a later section. Although it can be accepted that the erythrocytes and granulocytes are normally formed extravascularly in the marrow, this is not necessarily true of haemopoiesis in other situations. In the spleen, for instance, active haemopoiesis in dilated sinusoids may sometimes be seen in pathological material (see also the section on foetal erythropoiesis).

The Morphology of Normal Erythropoiesis

Nomenclature of the erythrocyte precursors.—For the sake of simplicity the vexed question of nomenclature* has so far not been considered, but the subject cannot be entirely avoided. The normal form of erythropoiesis gives rise to the normal erythrocyte (the normocyte) by a process best referred to as normoblastic erythropoiesis. The earliest definite erythrocyte precursor is the pronormoblast, and later nucleated forms are normoblasts. In later sections we shall refer to and describe other types of formation, megaloblastic, intermediate, and macronormoblastic, etc.

The term " erythroblast " should be reserved for any nucleated erythrocyte precursor without reference to the form of development, in accordance with the original usage of Ehrlich (Ehrlich and Lazarus, 1898, 1900).

Synonyms for the pronormoblast and normoblast.—Ferrata (Ferrata and Storti, 1948) called the earliest recognizable erythrocyte precursor the pro-erythroblast, derived from the haemocytoblast and developing through erythroblast stages to the erythrocyte. This terminology is used by the Italian school of haematology generally (Ferrata and Storti, 1948; Rastelli, 1943c). Naegeli (1931) has used the term macroblast (makroblast) to refer to the younger and larger erythroblasts, particularly in embryonal organs and in the marrow in states of rapid

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* Silver impregnation of the marrow, by demonstrating the reticular framework, helps to differentiate the marrow stroma with its network of fine fibrils from the sinusoids which lie on a layer of longitudinal argyrophil fibrils (see Plate 1, Figs. 5 and 6).

* In this paper we discuss the orthodox and traditional nomenclature only. Osgood and Ashworth (1937) introduced an entirely novel system which has not been generally accepted. The whole question is now once more under review by the International Society of Haematology.

blood regeneration, and Swiss authors such as Rohr (1940) and Leitner (1941) have used the term similarly, but this usage is confusing and better avoided (see the discussion on macronormoblasts in a later section). Similarly, the use by Sabin and co-workers of the term " megaloblast " for young basophilic red cell precursors under normal conditions (Doan and others, 1925) has no justification and has generally been abandoned.

Schulten (1937) derived the normal erythrocyte from the pro-erythroblast through macroblast and normoblast stages, and Dameshek and Valentine (1937) a series of normoblasts, A, B, and C, from haemohistioblasts through the erythrogone. Israëls (1939), whilst deriving the erythrocytes from multi-potent haemocytoblasts, called the earliest recognizable stage the pro-erythroblast, and the subsequent stages of normoblasts A, B, and C, in increasing order of maturity.

Turnbull (1936a), basing his observations on sectioned material, described the derivation of normoblasts from pluripotent haemocytoblasts through a series of "primary erythroblasts." He recognized three stages of normoblasts of similar size; the earliest with basophilic cytoplasm, then the polychromatic stage, and finally ripe orthochromatic normoblasts of the final stage.

Formation in intra-uterine life.—Details of the formation of the erythrocytes in intra-uterine life are of more than academic interest, for there are certain interesting contrasts and analogies between foetal erythropoiesis and that of the adult in health and in disease. Ehrlich (1880; Ehrlich and Lazarus, 1898, 1900) was the first to call attention to the similarity between the megaloblasts of pernicious anaemia and the haemoglobinized* primitive erythroblasts developing within the primitive blood vessels of the presomite human embryo of 2 to 5 mm. in length. These primitive foetal erythroblasts do not lose their nuclei and are very large cells. They are replaced by the first " definitive " erythroblasts, which in size and nuclear structure are intermediate between the cells of the primitive generation and those of adult life.† These cells lose their nuclei when mature and give rise to erythrocytes (macrocytes) whose volume is greater than those of the normal adult. The nucleated precursors may be termed macronormoblasts‡ (Jones, 1943). The liver is the chief site of their formation in mid-foetal life.§ From the fifth month of intra-uterine life onwards erythropoiesis commences within the marrow cavities of the ossification centres and at birth intramedullary formation is of prime importance, extramedullary foci having almost entirely disappeared by this time. The type of formation at birth is normoblastic and similar to that of the adult.

The cause of this variation in cell morphology at different age periods is far from clear. There is a gradual diminution in erythrocyte diameter and volume as the foetus matures,* but there is little evidence to prove that the macrocytosis is dependent upon an insufficient supply of anti-anaemia factors (Wintrobe, 1946a). Possibly the presence of the primitive persistently nucleated erythroblasts recalls phylogenetic development. · That foetal macrocytes may have a shortened life *in vivo* is suggested by Mollison's (1948) transfusion experiments. In this ` connexion it is interesting to recall that macrocytosis (and macronormoblasts) are seen in adult life in certain anaemias associated with rapid blood formation.

It is well known that foci of extramedullary haemopoiesis may be met with in adults in a variety of blood diseases, particularly where the bone marrow is widely invaded by carcinoma or where the marrow is undergoing fibrosis as in myelosclerosis. Less frequently extramedullary haemopoiesis is seen in haemolytic anaemia or even in pernicious anaemia. The foci occur most frequently in the liver and spleen, sites of vigorous intra-uterine formation, but are occasionally found in lymph glands or as heterotopic foci (see Vaughan, 1936a and b).

Normoblastic erythropoiesis in the adult.— The irregular lattice-like pattern of cellular and fatty areas in the adult human marrow has already been referred to, and close inspection of the cellular areas shows that focal areas of erythropoiesis are surrounded by larger zones of leucopoiesis.

The basic potentially haemopoietic cell in the marrow is a reticulum cell. Maximov (1927) has described the appearance of these cells in sections as flattened, pale-staining, and inconspicuous, and he considered them to be less differentiated than the dye-storing and phagocytic cells of the marrow stroma or the littoral cells of the marrow sinusoids. The question as to whether these more differentiated phagocytic cells can form haemocytoblasts or more mature forms of haemopoietic cells has not been settled and is discussed by Bloom (1938b). Ferrata and Storti (1948) recognized the haemohistioblast as an intermediate form between the primitive reticulum cells and haemocytoblasts. Clearly, irrespective of nomenclature, there is continuity of development between the most primitive and the more mature forms. Normally, these primitive cell types are present in small numbers and are somewhat difficult to identify in marrow smears. The cells are fragile and easily broken up by spreading, and cytoplasmic outlines may be indistinct. In pathological states such as Addisonian pernicious anaemia, and in certain refractory anaemias where there is hyperplasia of primitive cells, they are more frequent and may be seen in groups or even as syncitia (see Schleicher, 1945).

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* It appears that the type of haemoglobin produced at all stages of intra-uterine life is the same—that known as foetal haemoglobin (H. M. Jope, 1948; Hoch and others, 1949).
† The primitive generation of erythroblasts is formed intravascularly, as are the definitive erythroblasts (macronormoblasts) in early foetal life. Later, erythropoiesis in the liver is at first both intravascular and extravascular, but eventually is entirely extravascular, as it is in the bone marrow from the start (Gilmour, 1941).
‡ Some details of macronormoblastic development are given later in this paper.
§ Gilmour's (1941) paper is an important contribution to the study of intra-uterine haemopoiesis. It is based on 57 human embryos and foetuses. See also Bloom and Bartelmez (1940).

* Wintrobe (1946, p. 32) gives interesting data from thirty human foetuses; see also Wintrobe and Shumacker, 1935.

In film preparations the marrow reticulum cells vary between 20 and 30 μ. The cytoplasm is palely basophilic and abundant, and may contain a few azurophil granules; fine spherical mitochondria are numerous and evenly distributed. The nucleus is large, round, or oval, with a pale-staining* fine chromatin pattern and one or more round or oval nucleoli.

The haemocytoblast† is a large cell of somewhat variable outline, in size averaging about 20 μ in diameter. The cytoplasm is moderately deeply basophilic, and contains numerous mitochondria but no granules. The nucleus is round, averaging about 15 μ in diameter; it stains palely with Romanowsky dyes and presents a finely stranded and stippled (leptochromatic) chromatin pattern. Multiple nucleoli are present or a single elongated nucleolus of complex form bounded by a very fine layer of nucleolus-associated chromatin.

The pronormoblast is a slightly smaller cell than the haemocytoblast and usually of a rounded outline, although often showing fine pointed processes at the periphery of the cytoplasm. The average diameter is less than 20 μ and the nucleus less than 15 μ. The cytoplasm is more basophilic but less extensive than in the haemocytoblast; mitochondria are numerous. The chromatin pattern of the nucleus is more condensed and the nucleoli are smaller and are less easily seen, being surrounded by denser nucleolus-associated chromatin. The term *normoblast* refers to the developing erythroblast from the stage when the nucleoli have disappeared to the loss of the nucleus itself. As the cell matures there is a progressive shrinkage in size to about 8 μ and a parallel decrease in size of the nucleus. The cytoplasm becomes less basophilic as the cell matures, and increasingly acidophilic, this latter property being considered to be due to the formation of globin or haemoglobin.

Thus normoblasts may be described as basophilic, polychromatic, or orthochromatic, although in our experience fully orthochromatic cells are rarely encountered in normal subjects—that is to say, cells in which the cytoplasm is as acidophilic as that of adult erythrocytes. Such cells are, however, often illustrated (Wintrobe, 1946, plate 2; Whitby and Britton, 1946, plate 4). Everything depends upon the staining. It is possible to stain films to show orthochromatic normoblasts, but they are seldom seen if staining is controlled in such a way that polychromasia is well demonstrated. Indeed it is difficult to imagine an orthochromatic normoblast (having presumably lost its cytoplasmic basophilia) becoming a polychromatic reticulocyte and regaining cytoplasmic basophilia when its nucleus is disposed of. This would only be possible if the desoxyribonucleic acid of the normoblast nucleus contributed to the basophilic staining of the cytoplasm of the non-nucleated cell which developed from it, and for this there is no evidence. Brachet (1946) states, however, that Dustin suggested such a possibility. Reticulocytes are Feulgen-negative. If ribonucleic acid had been formed from desoxyribonucleic acid, an increase of both the reticular-filamentous material and diffuse basophilia removable by ribonuclease would be expected in the reticulocytes as compared with the normoblasts, but this does not seem to be so. A point that should be stressed is that the progressive diminution in size and pyknosis of a cell's nucleus is a more reliable guide to that cell's maturity than is the state of ripening of the cytoplasm, and that even in the normal the relative progress of nuclear and cytoplasmic ripening varies appreciably from cell to cell.

The areas of deeply staining chromatin, which first appear in the vicinity of the dwindling nucleoli and near the nuclear membrane, are brought together by shrinkage of the nucleus and ultimately fuse. In its final form the nucleus is entirely pyknotic and about 4–6 μ in size. That the changing pattern of the nuclear chromatin is not an artifact produced by fixation has been shown by studies with the electron microscope (Rebuck and Woods, 1948).

A few additional details as to the cytoplasm of erythroblasts may be added. Although the earlier forms possess a cytocentrum, Golgi apparatus, and mitochondria (Maximov and Bloom, 1948), these structures are lost in the course of differentiation. Mitochondria may be demonstrated by the supravital use of Janus green (Key, 1921; Sabin, 1928), and Jones (1947, 1948) has shown that, due to their content of lipoids, they can be stained by Sudan black by the method of Baker (1945) in air-dried films fixed in formol-calcium chloride (see Plate IV, Fig. 5). Jones has also used phase contrast techniques. He has demonstrated that in air-dried unstained films mounted in formalin, mitochondria lie above and below the nucleus, and that in this way they must contribute to the chromatin pattern of the nucleus

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* The descriptions given hereafter refer to Romanowsky-stained methanol-fixed dry film preparations. The appearances in sections or in wet fixed films differ due to the preservation of a more spherical shape of the cell and to the use of different fixatives. A description is outside the scope of this article.

† The haemocytoblast of Ferrata is the marrow stem cell of the erythrocytes, granulocytes, and megakaryocytes; it is the most organized stem cell capable of developing and differentiating to more than one type of blood cell (Ferrata and Storti, 1948; Rastelli, 1943c). Downey (1938a) discusses at length the potency of the haemocytoblast. He agrees that it is multi-potent, but unfortunately confuses the issue by referring to it as a "myeloblast." Helly's (1910) "erythrogone" is probably the same cell (see also Dameshek and Valentine, 1937). The multi-potency of the haemocytoblast is not universally accepted. Rohr (1940), for instance, derives the erythrogone (uni-potent) directly from the reticulum.

The development of the granulocytes from haemocytoblasts proceeds in a parallel fashion to that of the erythroblast. Mitosis occurs only in the earlier stages, and as the cell matures the degree of cytoplasmic and nucleolar basophilia diminishes and at the same time the azurophil and later the specific granulations appear.

as revealed by staining with Romanowsky dyes. He has also shown that the clear perinuclear area represents the unstained negative images of mitochondria and not hyaloplasm (see Wilson, 1928). More cytoplasmic particles are revealed by phase contrast microscopy than can be stained supravitally by Janus green.

Differential cell counts on marrow films.— Most workers perform differential cell counts on marrow films, and by presenting the data in the form of a " myelogram " express the incidence of the various cell types as percentages. Such figures, unfortunately, are less accurate than they appear to be at first sight. Not only is there an unknown admixture with cells from the peripheral blood in which the marrow cells are diluted, but in addition there is a tendency for the more primitive cells and relatively fixed marrow cells such as megakaryocytes to remain behind in the marrow. Ideally, differential counts should be performed on sections of aspirated material, but here, unfortunately, there is
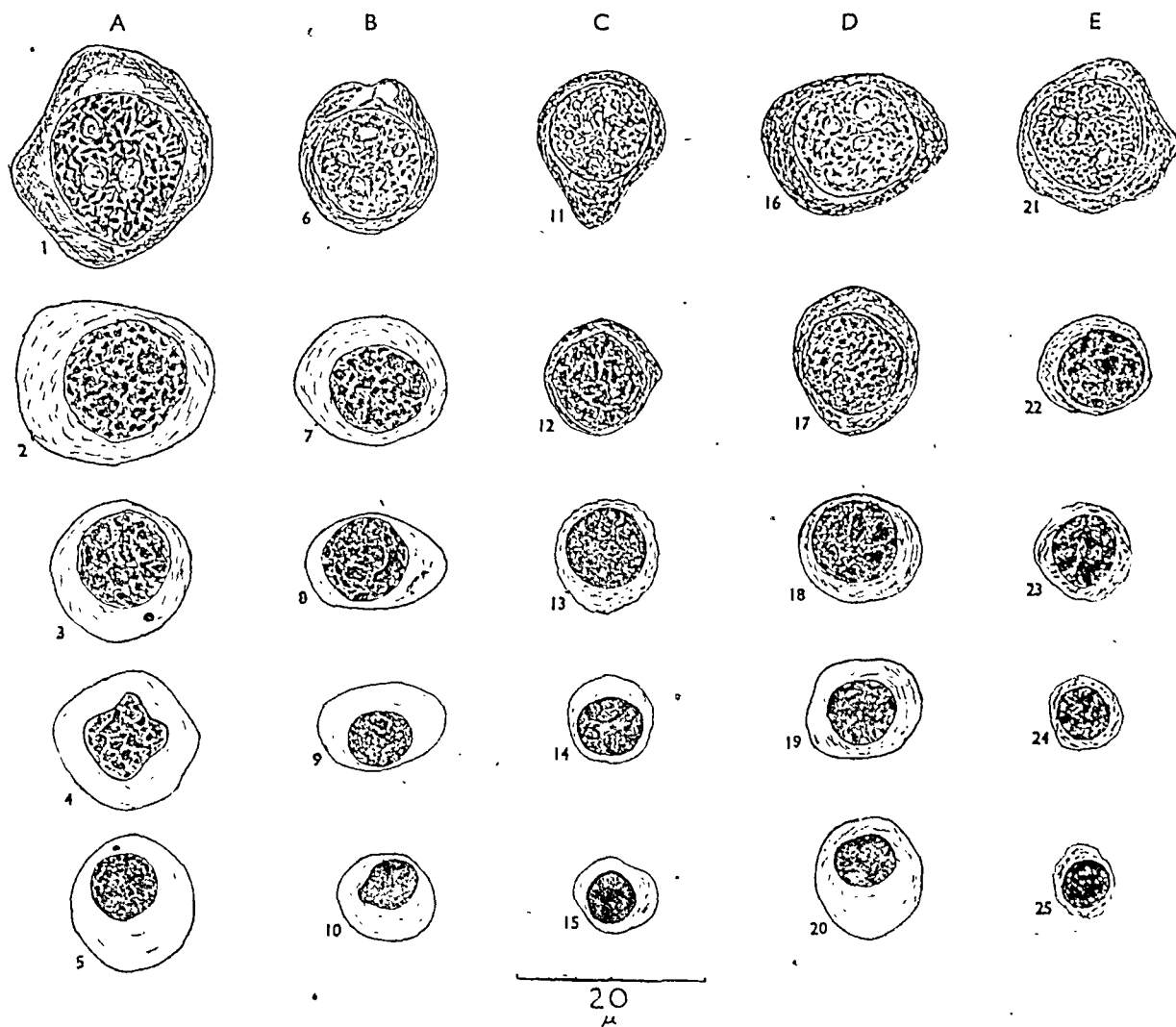
TEXT FIG. 1.—Series of scale drawings of maturing erythroblasts. Series A shows representative megaloblasts (Figs. 1–5) from a patient with severe Addisonian anaemia, erythrocytes 1,300,000 per c.mm. In series B (Figs. 6–10) are illustrated intermediate megaloblasts of comparable maturity from the marrow of a patient with steatorrhoea, erythrocytes 3,500,000 per c.mm. In series C (Figs. 11–15) the cells (normoblasts) were from the marrow of a normal adult man. Series D (Figs. 16–20) shows cells (macronormoblasts) of similar age groups from a patient with a macrocytic haemolytic anaemia (nocturnal haemoglobinuria), erythrocytes 1,500,000 per c.mm. In series E (Figs. 21–25) the cells were drawn from the marrow of a patient with a severe iron deficiency anaemia, erythrocytes 3,000,000 per c.mm.

In each series the topmost cell is a primitive cell (promegaloblast or pronormoblast).
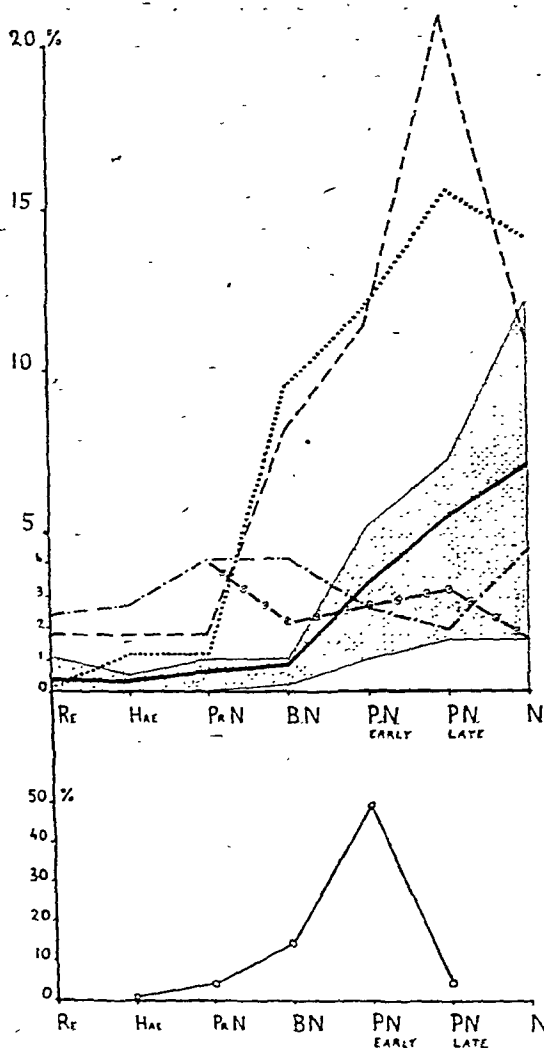
TEXT FIG. 2.—Curves of maturation and mitotic activity during erythropoiesis.

Upper figure: Maturation curves.

———— Maturation curve from calculated means of figures from 15 normal adult marrows (6 × 1,000; 9 × 500 cell counts). The observed range is indicated in the stippled area.

Maturation curve for erythropoiesis in Addisonian pernicious anaemia.

—·—·< ○—○— Normoblastic development.
         ·—·— Megaloblastic development.

— — — — Maturation curve in iron deficiency anaemia.

.... Maturation curve in congenital haemolytic anaemia.

Ordinates: Per cent of cell types.

Abscissae: Stages of erythropoiesis: RE, reticulum cells. HAE, haemocytoblasts. Pr.N., pronormoblasts. B.N., basophilic normoblasts. P.N. early and late, early and late polychromatic normoblasts. N., pyknotic normoblasts.

Lower figure: Mitosis during erythropoiesis.

Distribution by developmental stages of 67 erythroblasts in mitosis, encountered in 100 mitoses from normal marrows.

Ordinates: Per·cent of the mitotic cells.

Abscissae: Stages of erythropoiesis, as in upper figure.

difficulty in the proper identification of cells. Dameshek and others (1937) give illuminating figures for parallel observations made on sections and on smears made from puncture fluid, and other observations are reviewed by Osgood and Seaman (1944) and by Leitner and others (1949). Osgood and Seaman, in an excellent review, also discuss the unavoidable statistical errors involved in making differential counts and the necessity for counting large numbers of cells, particularly if an attempt is being made to assess the frequency of cells present in only small numbers. A further difficulty is the impossibility of accurately dividing into arbitrary classes cells of which every gradation of development

may be seen; and the fact that different authors naturally use different criteria to separate. say, " early " from " late " normoblasts makes comparison between their figures still more difficult.

For all the above reasons the figures given in the literature for the " normal " myelogram differ widely, as is illustrated in the tables of Bodley Scott (1939), Osgood and Seaman (1944), and Leitner and others (1949c).

Pontoni (1936) and Rastelli (1943d) have also discussed the analysis of differential cell counts. Pontoni correctly uses the term " haemomyelogram " for the ordinary differential count, and restricts the use of the term " myelogram " to counts

made on the residuum of cells after excluding the fully ripened ones, that is the segmented granulocytes, lymphocytes, and monocytes, etc. The commonly employed leucocyte-erythroblast or leucocyte-erythroid ratio, being based on the " haemo-myelogram," is of limited usefulness; in practice it gives a very wide range in normal subjects (2: 1 to 12: 1). That dilution with peripheral blood is a major factor in leading to a high leucocyte-erythroid ratio is shown by our lymphocyte values in a series of fifteen normal volunteers. The lymphocyte percentage varied between 13 and 31 per cent, and although some of these cells may have been derived from isolated lymph follicles, in most instances it is likely they came from the peripheral blood.

Pontoni's " leuco-erythrogenetic " ratio is a better expression of the relative proportions of leuco-poiesis to erythropoiesis. Mature leucocytes are excluded from the count, and in our series of normal subjects the ratio varied from 0.56: 1 to 2.67: 1. In most anaemias the ratio is below unity.

Fortunately, the factor of dilution with peripheral blood does not affect differential counts on marrow erythroblasts, unless there is a marked peripheral erythroblastaemia. These differential counts are more satisfactorily expressed by relating the numbers of the different classes of erythroblasts to the total number of erythroblasts rather than expressing them as percentages of the total nucleated cells present.

Because of differences in classification it is difficult to find from the literature normal ranges for the proportion of erythroblasts of different maturities. There is, however, wide agreement that in health the pronormoblasts and basophil normoblasts are few in number and that normoblasts of medium maturity with various grades of acidophilia of their cytoplasm and increasing density of the nucleus predominate. Fully pyknotic cells are less numerous, and pyknotic cells with fully orthochromatic cytoplasm are rarely met with. In our series of fifteen normal subjects, 2 per cent of the erythroblasts were pronormoblasts, 5 per cent basophilic normoblasts, 51 per cent early and late polychromatic normoblasts, and 42 per cent pyknotic normoblasts. In disease these proportions may be much altered.

Maturation curves (Pontoni, 1936; Baserga, 1939) have been used to illustrate the changes in proportion of the various classes of cells in different diseases or at different stages in the course of an illness (Text Fig. 2). Cotti and others (1938) have used a rather different way of expressing the proportion of primitive to maturing cells, and by their maturation index indicate the numbers of
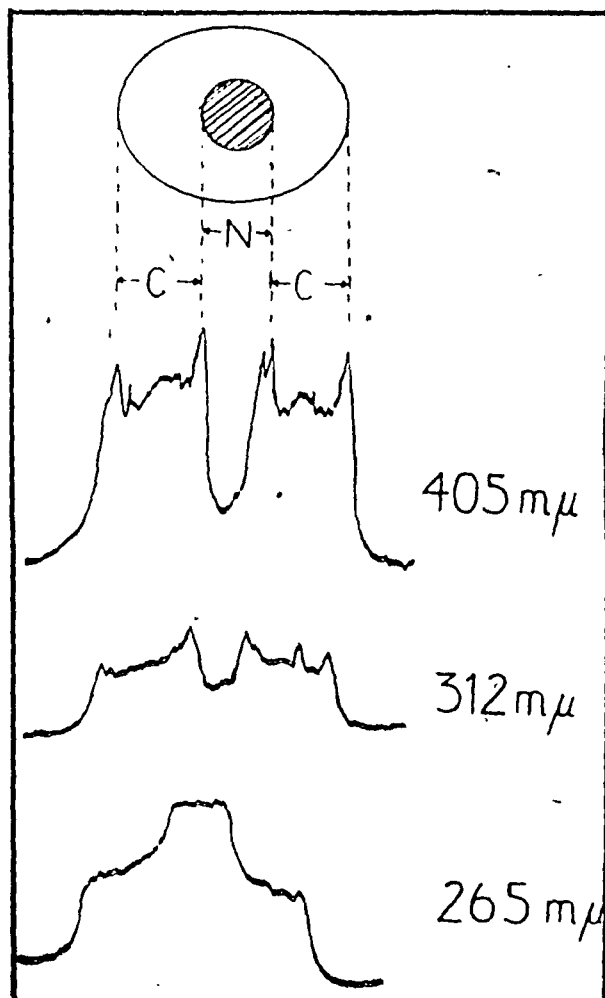
TEXT FIG. 3.—Densitometer records across spectrogram of frog red cell at three wavelengths, obtained from reflecting microscope. (Figure kindly supplied by Drs. R. Barer, E. R. Holiday, and E. M. Jope.)
N., cell nucleus. C., cytoplasm.
405 mμ. In the intense Soret band absorption due to haemoglobin.
312 mμ. A wavelength at which little specific absorption occurs.
265 mμ. In the strong absorption band due to purine and pyrimidine residues of the nucleic acids.

polychromatic and orthochromatic erythroblasts relative to the basophilic erythroblasts. We have employed a similar device to express the marrow immaturity in Addisonian and other megalocytic anaemias (Text Fig. 4).

Growth and Differentiation of Erythroblasts

In the previous sections we have described the stages in development from the haemocytoblast to the pyknotic normoblast. It is almost superfluous to state that this separation into stages is artificial;

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ERYTHROID-LEUCOCYTE RATIO

RATIO OF PRIMITIVE TO MATURING CELLS

ERYTHROCYTES PER C.MM.
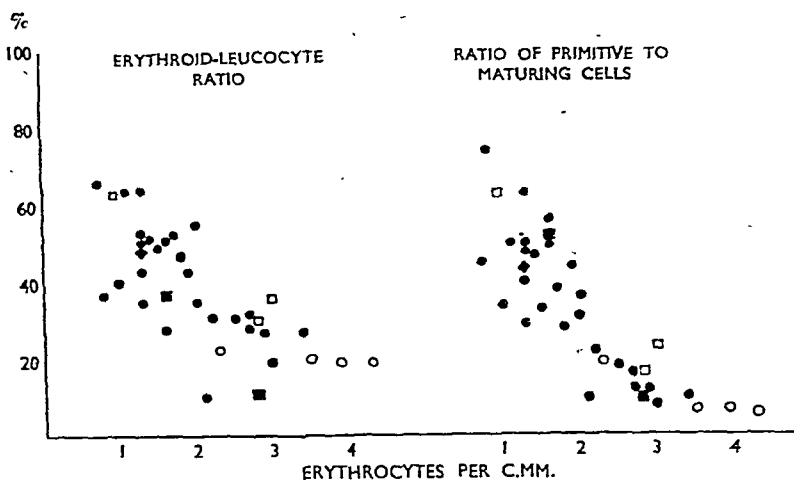
TEXT FIG. 4.—Relationship between the peripheral erythrocyte counts and the erythroid-leucocyte ratios (left-hand chart) and the primitive cell-maturing cell ratios (right-hand chart) in thirty-five patients who had megaloblastic marrows. Twenty-six of the patients had Addisonian pernicious anaemia, ●: four patients had steatorrhoea, ○; in three patients the anaemia was associated with pregnancy, □; in two with malnutrition, ■; and in one patient, ◆, anaemia had followed a gastro-enterostomy.

in reality there is a smooth gradation from the most primitive to the most mature forms. This process of development is a dual one, involving growth and cell division and differentiation. According to Cowdry's (1942) classification of levels of cellular activity the haemopoietic reticulum cells and haemocytoblasts are classed as vegetative intermitotic cells and the earlier erythroblasts as progressively differentiated intermitotic cells. In the final stages of development, when differentiation is completed, the cells are fixed post-mitotic cells.

Mitosis during erythropoiesis.—A proportion of the haemopoietic cells are always in mitosis. Japa (1942) studied the sternal marrows from three non-anaemic subjects and found 0.5 per cent of mitoses in fixed and stained films, but in aceto-carmine squash preparations from the same material there were 1.5 per cent, about 45 cells of the 100 in mitosis being erythroblasts. He thought that some of the dividing cells were disrupted when films were made. Mitoses may be seen at all stages, from haemopoietic reticulum cells to the polychromatic erythroblasts. Mitoses are not observed in latest polychromatic and pyknotic normoblasts, which is not surprising in view of the nuclear structure. Normally the absolute numbers of mitoses increase from the more primitive to the more mature stages, and it is rare to find a primitive cell in mitosis without extensive search. However, as the numbers of intermitotic cells also increase at each stage, the relative number of cells in mitosis at each stage is not so variable (see Text Fig. 2). This suggests an orderly increase in cell numbers by

mitosis throughout the growth and earlier differentiation phases of erythropoiesis. The products of the final mitoses then complete differentiation irreversibly. Abnormalities in mitosis leading to the formation of multinucleated cells are not infrequently found, even in normal erythroblasts and their precursors (see p. 30).

Fieschi (1938) represented the incidence of each stage of the mitotic cycle graphically and found that metaphases were the most numerous in normal erythropoiesis, and our results and those of Japa (1942) also indicate this. Increased erythropoiesis, however, leads to a higher proportion of prophases.

Life span of the erythroblasts.—The exact number of mitoses that take place in the development of a normal erythrocyte and the length of the mitotic and intermitotic periods at each stage of development are unknown. The whole process probably occupies several days. Ponder (1948) calculates that in the rat the lifetimes of the haemocytoblast, "erythroblast," normoblast, and reticulocyte are 0.23, 0.35, 2.3, and 3.6 days respectively. Hevesey and Ottesen (1945) have shown that if phosphates containing P^{32} isotope are fed to hens, isotope appears in the desoxyribonucleic acid of the nuclei of circulating erythrocytes after five days (see also Hevesey, 1948a). Shemin and Rittenberg (1946) have shown that in normal man N^{15}-containing haem appears in circulating erythrocytes within two days of the ingestion of N^{15}-glycine. This represents the time between the formation of haem and the delivery of the erythrocytes into the blood stream, and is only part of the total time required for erythropoiesis (see also p. 21).

In man there is other indirect evidence. The response to potent liver extract in a severe case of Addisonian pernicious anaemia indicates that the reticulocytes are delivered into the peripheral blood stream at the maximum rate at about four to six days after an injection of liver. This period of maximum reticulocyte production corresponds presumably to the final maturation of the predominating age group of marrow cells, which in a severe case are the abnormal haemocytoblasts and basophilic promegaloblasts. Owren's (1948) studies on the mechanism of the production of crises in congenital haemolytic anaemia also provide some evidence about the rate of development of the maturing erythroblast. In his case 4, regeneration of "pro-erythroblasts" (pronormoblasts) in the marrow had started on the ninth day of the crisis. Reticulocytes were appearing in the

NORMAL AND ABNORMAL ERYTHROPOIESIS

Figs. 1 to 14: Normoblastic Erythropoiesis in Normal Sternal Marrow

Fig. 1.—Haemohistioblast (haemopoietic reticulum cell). Fine azurophil granulation of palely basophilic cytoplasm. 22.7 × 13.2 μ; nucleus 13.7 × 10.5 μ.

Fig. 2.—Haemocytoblast, 19.2 × 17.5 μ; nucleus 15.0 × 13.4 μ.

Fig. 3.—Pronormoblast, 13.3 × 12.5 μ; nucleus 12.5 × 10.8 μ.

Fig. 4.—Basophilic normoblast, 12.5 × 11.7 μ; nucleus 10.8 × 10 μ.

Fig. 5.—Early polychromatic normoblast, 10.8 × 10.8 μ; nucleus 8.3 × 7.5 μ.

Fig. 6.—Late polychromatic normoblast, 9.2 × 8.3 μ; nucleus 6.6 × 6.6 μ.

Fig. 7.—Pyknotic normoblast, 8.3 × 7.3 μ; nucleus 5 × 4 μ.

Fig. 8.—Mature erythrocyte, 7.8 × 7.2 μ.

Fig. 9.—Haemocytoblast, 13 × 10.4 μ; nucleus 9.1 × 7.8 μ. Film wet-fixed in Susa; pyronin-methyl green staining after one hour in veronal-acetate buffer, pH 6.85. Deep cytoplasmic and nucleolar basophilia attributable to content of ribonucleic acid.

Fig. 10.—Polychromatic normoblast (7 × 7 μ; nucleus 6.3 × 5.6 μ) from wet-fixed film in Susa stained by Feulgen method. Chromatin stains purple. Cytoplasm is acidophilic and stains with light green yellowish.

Fig. 11.—Polychromatic normoblast (8.4 × 6.3 μ) from same film as Fig. 9. Cytoplasmic basophilia is much less at this stage; nucleoli have disappeared and chromatin nodes stained by both dyes are prominent.

Fig. 12.—Polychromatic normoblast (7.7 × 7.7 μ) from similar film treated with ribonuclease in veronal-acetate buffer, pH 6.85, for one hour before staining. Basophilic material stainable by pyronin extensively removed from cytoplasm by enzyme. Chromatin stains more markedly with methyl green through removal of pyronophil component by enzyme.

Fig. 13.—Late polychromatic normoblast (7.0 × 7.0 μ) from brilliant cresyl blue preparation of normal marrow; wet-fixed film in Susa treated with pH 6.85 buffer and stained by pyronin-methyl green. The basophilic material of the cytoplasm is aggregated by action of the vital dye.

Fig. 14.—Similar cell from parallel preparation treated with ribonuclease at pH 6.85: this has removed the aggregated pyronophil material.

Figs. 15 to 21: Intermediate Erythropoiesis in Sternal Marrow of a Patient with Mild Addisonian Pernicious Anaemia

Fig. 15.—Haemopoietic reticulum cell developing to haemocytoblast, 31.7 × 21.7 μ; nucleus 20 × 18.4 μ.

Fig. 16.—Intermediate promegaloblast, 20 × 18.4 μ; nucleus 15.5 × 15.5 μ.

Fig. 17.—Basophilic intermediate megaloblast, 16.7 × 16.7 μ; nucleus 13.4 × 13.4 μ.

Fig. 18.—Early polychromatic intermediate megaloblast, 16.7 × 14.2 μ; nucleus 11.7 × 11.7 μ.

Fig. 19.—Late polychromatic intermediate megaloblast, 11.7 × 10 μ; nucleus 6.7 × 6.7 μ.

Fig. 20.—Pyknotic intermediate megaloblast, 11.3 × 11.3 μ; nucleus 3.3 × 3.3 μ.

Fig. 21.—Intermediate megalocyte, 9.6 × 8 μ.

Figs. 22 to 34: Megaloblastic Erythropoiesis in Sternal Marrow of a Patient with Severe Addisonian Pernicious Anaemia

Fig. 22.—Haemopoietic reticulum cell (haemohistioblast), 32.5 × 25 μ; nucleus 21.7 × 16.7 μ.

Fig. 23.—Haemocytoblast, 21.7 × 20.7 μ; nucleus 18.4 × 16.7 μ.

Fig. 24.—Promegaloblast, 23.4 × 23.4 μ; nucleus 18.4 × 18.4 μ.

Fig. 25.—Basophilic megaloblast, 20.0 × 16.7 μ; nucleus 15.0 × 14.3 μ.

Fig. 26.—Early polychromatic megaloblast, 21.7 × 21.7 μ; nucleus 15.8 × 12.5 μ.

Fig. 27.—Late polychromatic megaloblast, 19.2 × 19.2 μ; nucleus 13.2 × 10 μ.

Fig. 28.—Pyknotic megaloblast, 16 × 13 μ; nucleus 6.7 × 5 μ.

Fig. 29.—Megalocyte, 10.2 × 9.6 μ.

Figs. 30 to 34: Cells from Films of Sternal Marrow in Addisonian Pernicious Anaemia. wet-fixed in Susa and stained by Pyronin-methyl Green after Treatment with Veronal-Acetate Buffer, pH 6.85, or Buffer-ribonuclease for one Hour

Fig. 30.—Haemocytoblast, 15.6 × 11.7 μ; nucleus 10.4 × 9.7 μ. Control slide in buffer. Deep pyronin staining of cytoplasm and nucleoli attributable to ribonucleic acid.

Fig. 31.—Haemocytoblast, 13.0 × 12.3 μ; nucleus 11.0 × 9.1 μ. Slide treated with ribonuclease. Extensive removal of pyronophil material by enzyme.

Fig. 32.—Early polychromatic megaloblast, 18.2 × 9.1 μ; nucleus 9.1 × 7.8 μ. Control slide in buffer. Nucleoli still present. Pyronophil basophilic material in cytoplasm is smaller in amount at this stage.

Fig. 33.—An older polychromatic megaloblast, 14.3 × 14 μ; nucleus 10.4 × 8.4 μ from same slide as Fig. 32.

Fig. 34.—A late megaloblast, 11.7 × 10.4 μ; nucleus 6.5 × 6.5 μ. Nucleoli absent and cytoplasm only slightly pyronophil.

Figs. 1 to 8 and 15 to 29 are drawn from air-dried films fixed in methanol and stained by May-Grünwald-Giemsa method.

All cells drawn using Zeiss 2 mm. Homog. Immers. Apochromat. (apert. 1.40) and × 8 compensating ocular, with 250 mm. tube length.
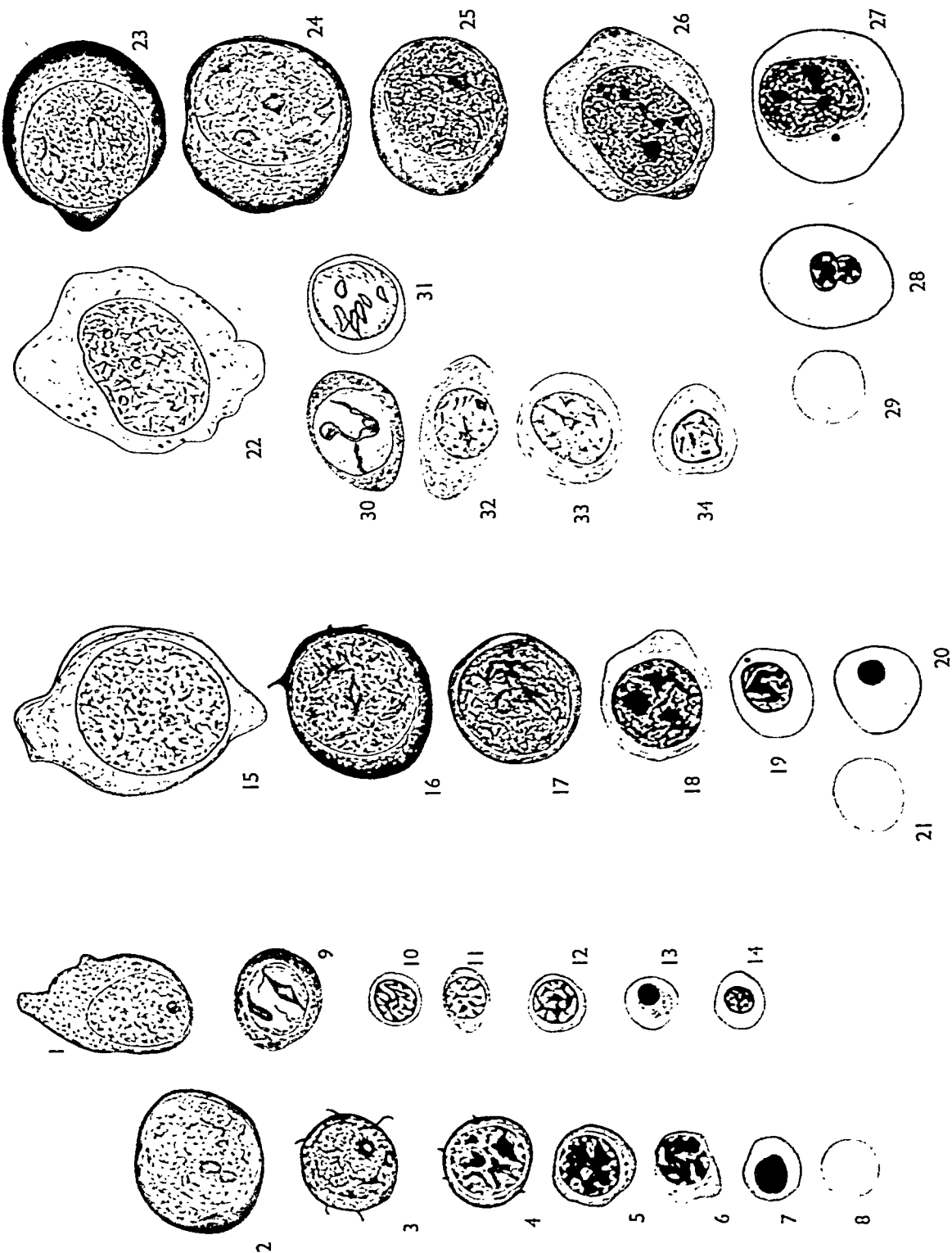
peripheral blood at a maximum rate between the twelfth and fourteenth days, giving a maturation time of three to five days.

The orthodox view that a single normoblast gives rise to a single erythrocyte has not passed unchallenged. Plum (1947) has maintained that the numbers of erythroblasts in mitosis are too small to yield the requisite daily quota of erythrocytes necessary to maintain equilibrium, and has published details of studies *in vitro* in support of his contention. He believes that several erythrocytes may be derived from each normoblast by budding off (gemmation) from the cytoplasm. Boström (1948) supports Plum's view and illustrates schematically how the budding process is supposed to take place (see also p. 30). These interesting and revolutionary views need confirmation.* Ponder (1948) on theoretical grounds does not see the necessity for gemmation, and it cannot necessarily be assumed that " erythroblasts " behave *in vivo* as they do *in vitro* in artificial culture.

In our experience study of biopsy films of normal or hyperplastic normoblastic marrows yields no support for the mechanism of gemmation. The general appearance of films seems to indicate that ripe normoblasts *become* reticulocytes after the nucleus is lost. If, for instance, an actively erythropoietic marrow film from a patient with haemolytic anaemia is examined, satisfactory evidence of budding from the cytoplasm is extremely difficult to find. On the other hand pyknotic normoblasts with well formed evenly rounded polychromatic cytoplasm are frequent. Some normoblasts with scanty irregularly contoured polychromatic or slightly basophilic cytoplasm may be seen, and at first sight it would appear possible that these were cells which had already budded off cytoplasm. Against this hypothesis is the serious objection that the only non-nucleated masses of cytoplasm usually seen are fully formed polychromatic erythrocytes, which seem to be clearly derived from pyknotic nucleated precursors. Basophilic and irregularly formed cytoplasmic masses are rarely found in normal or hyperplastic normoblastic marrows. What happens to the normoblasts with scanty cytoplasm is far from clear: possibly they never mature at all. The possibility of limited gemmation appears more plausible in marrow films from patients with severe megalocytic anaemias. Promegaloblasts and polychromatic megaloblasts of irregular contour are not infrequent, and separated fragments of cytoplasm may be found; whether these fragments can develop into mature erythrocytes is unknown.

Derivation of reticulocytes from normoblasts.—It is generally considered that reticulocytes are direct successors of ripe normoblasts, after loss of the nuclei. The evidence for reticulocytes being younger than the bulk of the circulating erythrocytes is firmly based on the constant finding of increased numbers of reticulocytes whenever other evidence indicates an increased production of erythrocytes, as well as upon the fact that blood from the bone marrow contains a higher proportion of reticulocytes than is found in the peripheral

blood (Ungricht, 1938). Moreover, culture *in vitro* (Jacobsen, 1947) and survival studies on transfused blood containing a large number of reticulocytes have demonstrated loss of basophilic material and transformation into non-reticulated erythrocytes (Young and Lawrence, 1945).

Reticulocytes are on the average probably a little larger in diameter, and possibly a little thinner, than are adult erythrocytes, and it is presumed, therefore, that a slight alteration in corpuscular shape takes place after the cells have left the bone marrow. It is possible that it is the spleen which is largely responsible for the modification of corpuscular shape (Miller and others, 1942); however, the evidence can be interpreted in more than one way.*

The characteristic feature of reticulocytes is their ability to react supravitally with certain basic dyes; they contain a component which is precipitated in unfixed corpuscles by brilliant cresyl blue as a blue granular network, and which in methanol-fixed air-dried films gives a diffuse basophilic staining and an appearance of " polychromasia " to the whole field of corpuscles. Dustin (1944, 1946) has shown that this basophilic material contains ribonucleic acid, and it seems very likely that it represents the last remnants of the basophilic ribonucleoproteins so abundantly present in the primitive cells from which the reticulocyte has been derived. Indeed, if material aspirated from the bone marrow is treated with brilliant cresyl blue before smears are made and fixed, staining with Romanowsky dyes will then show that the basophilic material in the primitive cells and in the developing normoblasts has been aggregated by the dye in much the same way as in the reticulocytes themselves. Another apparent difference between reticulocytes and adult erythrocytes is that reticulocytes contain more protoporphyrin than do the adult corpuscles† (Watson and Clarke, 1937; Watson and others, 1944). Haemoglobinizing normoblasts also appear to contain free protoporphyrin (Stasney and McCord, 1942).

It is as yet uncertain whether all the erythrocytes that enter the blood stream are unripened reticulocytes or whether a proportion are fully ripened adult erythrocytes. Heath and Daland (1930), Young and Lawrence (1945), and Wintrobe (1946) believe that a proportion of mature corpuscles are delivered. Baar and Lloyd (1943) and Nizet (1946) disagree with this. Nizet's view that all the cells delivered are reticulocytes is based upon a consideration of the constant proportion of the most mature type of reticulocyte in the blood stream compared with more immature types, and upon cross-circulation experiments with marked Heinz-body-containing

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* Several other heterodox hypotheses have been put forward (see *Lancet*, March 29, 1947).

* There is little question that reticulocytes seen in pathological states, such as in recovery from haemorrhage and in some haemolytic anaemias, are macrocytes with an increased diameter and volume (Dameshek and Schwartz, 1940; Wintrobe, 1946b). It is probably a fair inference that normal reticulocytes are also a little larger than adult corpuscles, but more evidence is required on this point. The controversial question as to how the spleen influences erythropoiesis is discussed by Doan and Wright (1946) and Dameshek and Estren (1947).

† The reticulocyte protoporphyrin cannot be held responsible for the reaction with cresyl blue as suggested earlier by Watson and Clarke (1937).

erythrocytes. The question is important in relation to calculations of the longevity of the erythrocytes based on reticulocyte ripening times, and cannot yet be considered as finally settled.

Loss of the nucleus of the normoblast.—All authors agree that the nucleus of the normoblast disappears after it becomes pyknotic* and the cell is ripe. The mechanism of its disappearance has been the subject of much discussion. There is no unanimity; loss by expulsion, karyorrhexis, or karyolysis have from time to time been thought to play a part. Naegeli (1931) considered that karyolysis took place, and states that this also was the view of Koellicker and Neumann. Amongst others in agreement with this conception are Cooke (1930), Davidson (1930), Habelman (1940), Kracke (1941), and Ferrata and Storti (1948). Howell (1890) thought on the other hand that expulsion was the mechanism, and more recently Maximov (1927), Turnbull (1936b), and Wintrobe (1946) support this point of view. Fieschi and Astaldi (1946) claim to have observed this in their marrow cultures.

It has been argued that the formation of Howell-Jolly bodies indicates that karyorrhexis does in fact take place, and that in certain circumstances it is incomplete and that a remnant of the nucleus persists. However, study of the bone marrow in those cases where Howell-Jolly bodies are being formed shows that they are separated off from the nucleus at a relatively early stage, before the nucleus as a whole becomes pyknotic. Moreover, this form of karyorrhexis is not normally seen, and it seems, therefore, unlikely that the nucleus of the normal normoblast is disposed of by this mechanism.

La Cour (1944) and Discombe (1946) have observed budding of nuclear material into the cytoplasm of animal and human polymorphonuclear leucocytes, and La Cour has suggested that the normoblast nucleus may lose its capacity to stain owing to loss of desoxyribonucleic acid during haemoglobin formation. It is interesting to note that in 1912 Schilling-Torgau suggested that it was only the chromatin of the normoblast nucleus that was lost. It seems almost unnecessary to point out that the presence or absence of a nucleus is usually judged by the presence or absence of chromatin staining and that loss of the nucleus itself would be inferred if for any reason its characteristic staining reactions were lost. *In vitro*, for example, depolymerization of nuclear desoxyribonucleic acid by the enzyme, desoxyribonuclease, and loss of staining has been shown by Brachet and Shaver (1948) to take place very rapidly.

It is true that in most smears of bone marrow cells naked pyknotic nuclei are occasionally found. Because it is impossible to exclude mechanical trauma as the cause, this observation by itself cannot be used to support the view that loss by expulsion occurs *in vivo*, especially in view of the small number of free nuclei encountered.

It is clear that no answer can yet be given as to how the normoblast loses its nucleus. The absence of transition stages between pyknotic normoblasts and reticulocytes seems to us significant; whatever the process, it is clearly a rapid one.

Regulation of erythropoiesis.—In health, erythropoiesis is nicely balanced to compensate for the normal daily loss of corpuscles, amounting to about 1/120 of the total of the circulating erythrocytes. Biopsy of the bone marrow gives a still picture of this normal compensatory erythropoiesis, but tells little of the mechanisms by which it is controlled and adjusted to the needs of the body. The very many haemopoietic factors which seem to be required are well reviewed by Cartwright (1947). These include several metallic elements (Schultze, 1940), amino-acids, vitamins, and growth factors, many of them being nutrients in a general sense. Knowledge of their importance is based upon critical experiments with laboratory animals and observations on man. A more mysterious control may be effected by endocrine glands and growth or maturation substances of intrinsic origin. Clinical evidence suggests that anaemia may develop in hypothyroidism (Bomford, 1938), in hypopituitarism (Snapper and others, 1937; Watkinson and others, 1947), in hypogonadism (McCullagh and Jones, 1942), and in Addison's disease (Wintrobe, 1946c). In rats, hypophysectomy regularly results in anaemia (Crafts, 1946). Finkelstein and others (1944) have claimed that male hormones stimulate and female hormones depress erythropoiesis. It seems reasonable to conclude that the endocrine glands control erythropoiesis to some extent, perhaps largely by their action on the growth and metabolism of the body as a whole. The subject is reviewed by Daughaday and others (1948).

The reticulocyte-ripening factor of Plum (1942) has already been mentioned, and Jacobsen (1947) refers to unpublished work suggesting that the ripening of normoblasts may be controlled by similar humoral factors. These interesting studies require confirmation.

A lowered oxygen tension has for long been considered an important stimulus to erythropoiesis, and the polycythaemia which develops at high altitudes is evidence of this relationship. The recent studies of Hurtado and others (1945) indicate that although there is a wide variation in response, the majority of people residing at high altitudes develop polycythaemia. There seems a direct relationship between the degree, duration, and continuity of the anoxia and the rise in the erythrocyte count.

The exact way in which oxygen-lack stimulates erythropoiesis is obscure. Grant and Root (1947), working with dogs, observed increased erythropoiesis to last for three weeks following loss of 30 per cent

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* Pyknosis of the nucleus denotes in general an injured or dying cell. M. J. D. White (1947), discussing pyknosis, suggests that this process may be a modified non-functional mitosis in which the chromosomes have become fused.

of the blood volume, but the lowered oxygen tension and saturation in the bone marrow blood lasted three to five hours only; and the same degree of increased erythropoiesis resulted when the blood loss was brought about by smaller successive bleedings, which did not result in any diminution in the oxygen tension (*see also* Grant, 1948 ; Berk and others, 1948). Again the studies made by Rosin and Rachmilewitz (1948) on marrow cultures *in vitro* indicated *depression* of erythropoiesis at oxygen percentages below 12 per cent and *increased* activity when the oxygen percentage was raised to 50 per cent. These results are not, however, supported by the clinical observations of Reinhard and others (1944), who observed a diminution in erythropoiesis, resulting in a fall in reticulocyte percentage and a fall in the erythrocyte count, when patients with sickle-cell anaemia inhaled 70 to 100 per cent of oxygen for eight to 20 days. Tinsley and Moore (1948) reported an extension of this work. They observed depression of erythropoiesis in patients with sickle-cell anaemia and with congenital haemolytic anaemia who inhaled 50 to 95 per cent oxygen, and a rapid rise in reticulocytes when oxygen inhalation was discontinued. The reticulocyte response of patients with Addisonian pernicious anaemia treated with liver and inhaling 70 to 80 per cent oxygen was also partly suppressed; a second and higher response followed the cessation of oxygen inhalation.

The picture is thus complex; reduced oxygen tension stimulates and increased oxygen tension inhibits erythropoiesis. Increased erythropoiesis regularly follows haemorrhage, although (in dogs) no reduction in oxygen tension may be demonstrable, and in marrow cultures reduced oxygen tension is a depressant rather than a stimulant.

It is obvious that further information is required on the relationship between plasma oxygen tension, the respiration of erythroblasts, and erythropoiesis. Possibly the actual flow of blood through the marrow is a factor which affects erythropoiesis, by controlling the delivery of erythrocytes into the circulation from the sinusoids, and perhaps even by affecting erythropoietic activity itself.

Cytochemistry of Erythrocyte Development

Before discussing what is known of the chemical changes which take place in the developing normoblast, it is necessary to refer briefly to recent knowledge in general cytochemistry, for in many respects the developing haemopoietic marrow behaves in the same way as do other actively growing tissues.

Although the factors which control the growth and differentiation* of cells remain obscure, more is known of some of the chemical substances involved, and of the chemical changes which take place during differentiation. The nucleic acids† play an important role in this process. They are biologically distributed in two chemical forms, the desoxyopentose polynucleotides or " desoxyribonucleic acid " and pentose polynucleotides or " ribonucleic acid." Caspersson and his school (consult Caspersson and Santesson, 1942; Caspersson, 1946; Thorell, 1947a) have studied the distribution of these two forms of nucleic acids by means of their ultra-violet spectral absorption. This is revealed from a sequence of monochromatic photographs or photometer readings obtained from a quartz microscope illuminated through a monochromator.

Both cell nuclei and the cytoplasm of young, actively growing cells can be shown to contain nucleic acids in high concentrations (Caspersson and Schultz, 1939, 1940). Both forms of nucleic acid are found. The nucleolus and cytoplasm contain ribonucleic acids associated with proteins; the Feulgen reaction* is negative in these areas of the cell. The nucleolus may also contain protein of the histone type.† The chromatin and chromosomes contain, however, desoxyribonucleic acid, and are Feulgen-positive; they also contain proteins.

Caspersson (1946) has suggested a number of principles which he believes to be of wide applicability: that protein synthesis needs the presence of nucleic acids; that the nucleus is the centre of the cell for the formation of proteins and that the nucleolus-associated chromatin secretes into the nucleolus substances of protein nature which diffuse outwards to the nuclear membrane; and that it is on the outer surface of the nuclear membrane that ribonucleoproteins are synthesized.‡§

Brachet and his school have approached the subject independently from a different angle. They have utilized enzymes (nucleases) to depolymerize nucleic acids from fixed cells and have judged their effects by alterations in the staining reactions, particularly with pyronin-methyl green. Purified ribonuclease has been used by Brachet (1942, 1946) and Brachet and Shaver (1948), and desoxyribonuclease (McCarty, 1946) has been employed to depolymerize desoxyribonucleic acid (Brachet, 1942; Sanders, 1946; Brachet and Shaver, 1948; Catchside and

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* The Shorter Oxford Dictionary defines " differentiation " (biol.) as: " The process, or the result of the process, by which in the course of development a part, organ, etc., is modified into a special form, or for a special function; specialization; also the gradual production of differences between the descendants of the same ancestral types." (See also Bloom, 1937.)

† Extensive treatment of the whole subject of the role of the nucleic acids is given in two recent symposia (1946, 1947) and in the reviews of Greenstein (1944) and Hevesey (1949b).

* The Feulgen reaction (Feulgen and Rossenbeck, 1924) is generally held to be a specific cytochemical test for desoxyribonucleic acid (Dodson, 1946; Stowell, 1946), although this has been questioned (Carr, 1945; Stedman and Stedman, 1947). The chemistry of the reaction has been investigated by Stacey and others (1946).

† The view that histones are important nucleolar constituents has recently been challenged (Pollister and Ris, 1947; see also Stedman and Stedman, 1947).

‡ Spiegelman and Kamen (1947) have warned against necessarily accepting the conception of a particularly close association between the formation of protein and the metabolism of nucleic acids.

§ Hinshelwood (1946) also reviews the conditions under which proteins are synthesized.

Holmes, 1946). The results of this work are very similar to those of Caspersson. Brachet (1946) considers, however, that chromatin, especially the heterochromatin,* contains a proportion of ribonucleic acid (see also Kauffman and others, 1948) and that the nucleus is of minor importance in relation to the synthesis of cytoplasmic protein compared with the " cytoplasmic particles " (mitochondria and microsomes) with which the ribonucleoproteins are largely associated (Bensley, 1942; Claude, 1943; Brachet, 1946).

The cytochemistry of the developing bone marrow cells has been studied by several authors. Thorell (1947b, etc.) has developed Caspersson's techniques of ultra-violet absorption microspectrophotometry. Brachet (1942) has applied his ribonuclease test to various amphibian and mammalian bone marrows. White (1947) has extended Brachet's work to normal and pathological human marrow cells, and J. N. Davidson and others (1948) have used the ribonuclease test as well as chemical microanalysis of marrow nucleic acids. Dustin (1946, etc.) has employed ribonuclease in his researches on reticulocytes and Wislocki and Dempsey (1946) have subjected samples of Rhesus monkey marrow to ribonuclease in addition to other histochemical techniques.

Thorell's work, recently summarized in a monograph (Thorell, 1947b), deserves further consideration. He worked with the marrow of rats and rabbits, and with normal and pathological human marrow. Individual living cells were subjected to analysis at different growth stages and the concentrations and proportions of nucleic acids, proteins, and haemoglobin were determined by means of their characteristic absorption maxima at particular wavelengths of the ultra-violet.*

Thorell's work has provided examples of the more general views of Caspersson. The primitive pronormoblasts contain the maximum amount (5 per cent approximately) of ribose polynucleotides in the cytoplasm and nucleoli. Desoxyribonucleic acid is confined to the nuclear chromatin and nucleolus-associated chromatin. The concentration of ribose

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* Heterochromatin (Heitz, 1929) is chromatin closely associated with the nucleolus and is thought to possess important metabolic functions (see Schultz, 1947). It is probably identical with the nucleolus-associated chromatin of Caspersson, which Thorell (1947b) believes may regulate the growth of marrow cells, as it does of cellular growth in general.

* The nucleic acids give a maximum extinction with light of wavelength 257 mμ. Proteins were estimated by absorption maxima at 280 mμ, attributable to their content of tyrosine and tryptophane (Holiday, 1936), and haemoglobin was estimated by absorption maxima at 404.7 mμ and 435.8 mμ (the Soret band).

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

B PLATE I.
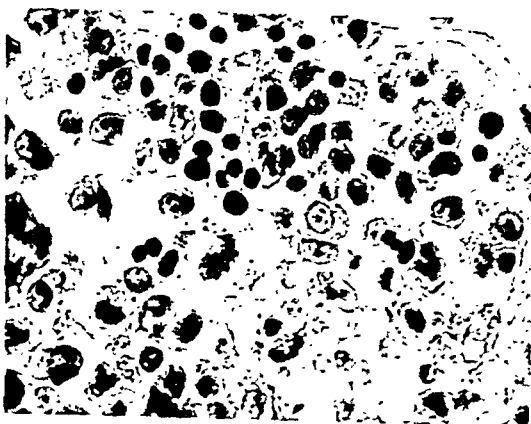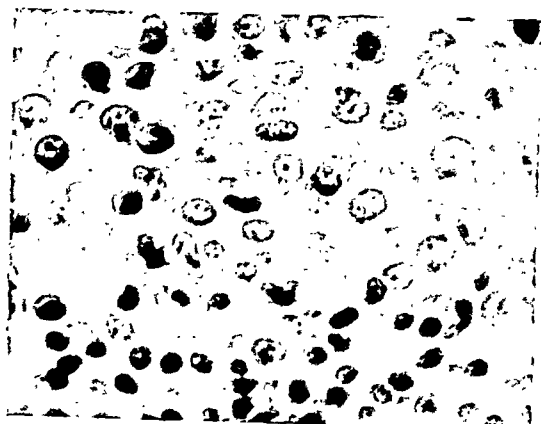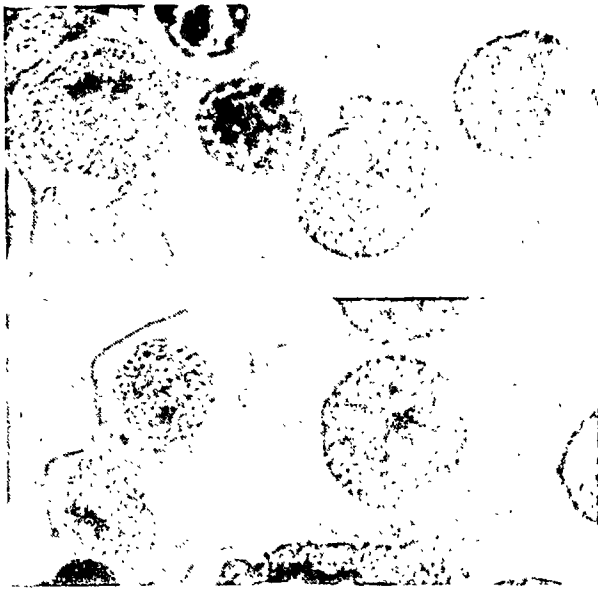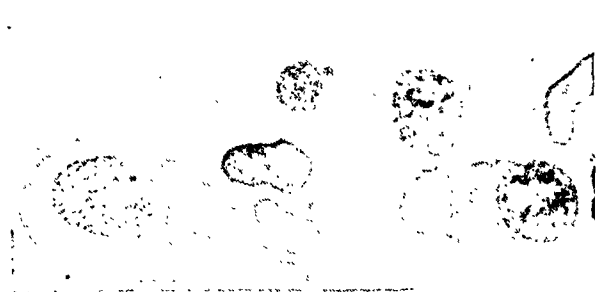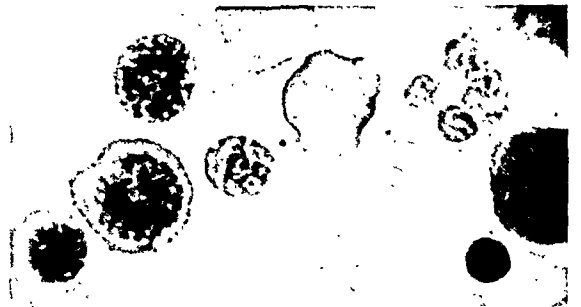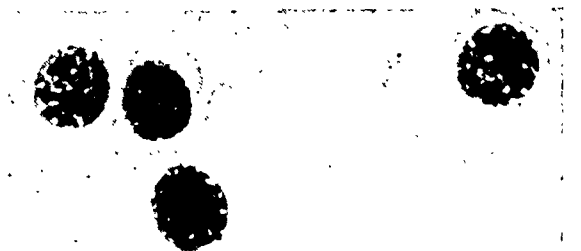
Fig. 1.

Fig. 2.
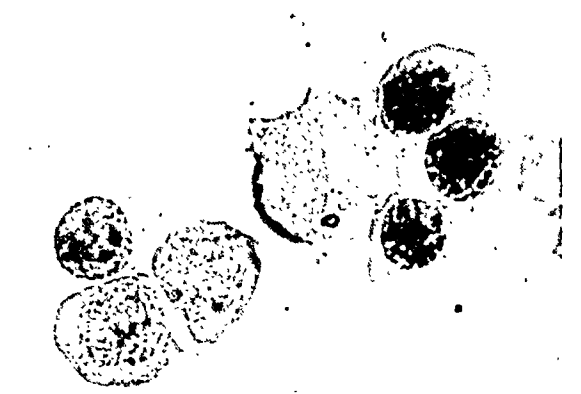
Fig. 3.

Fig. 4.

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Fig. 7.

Fig. 1.

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PLATE III.
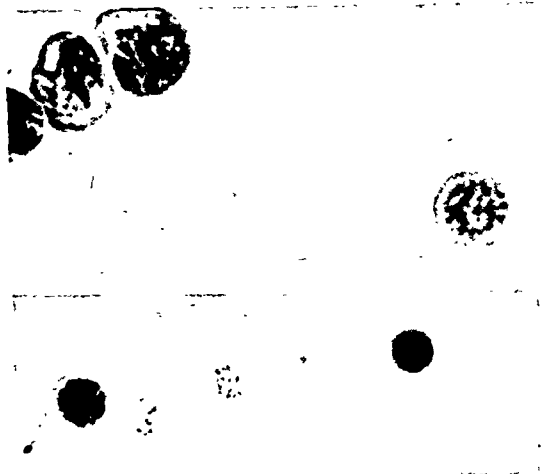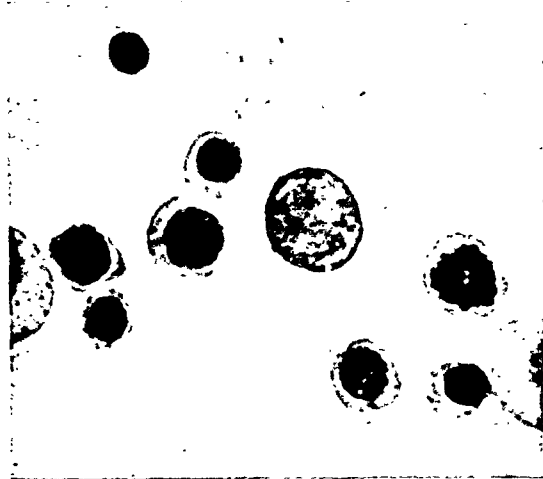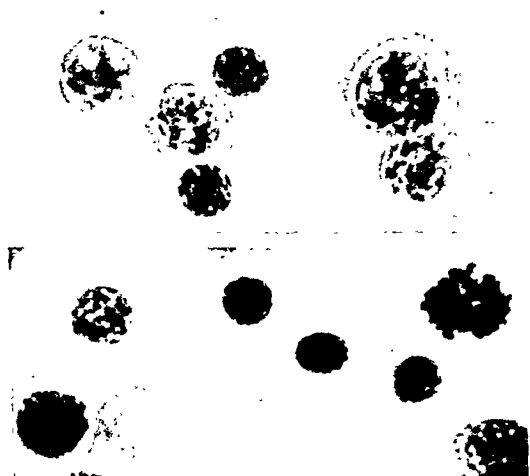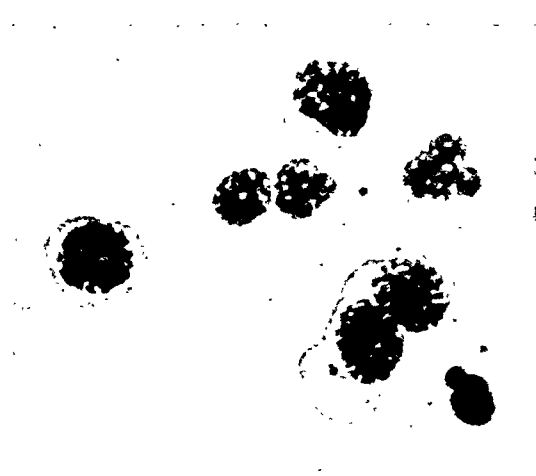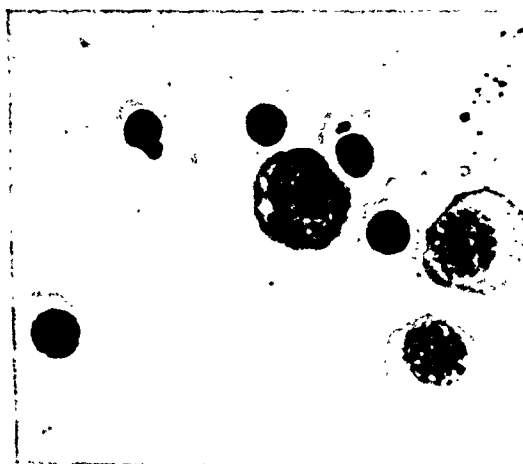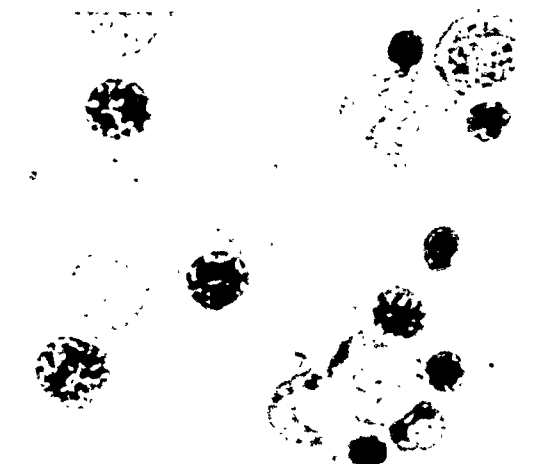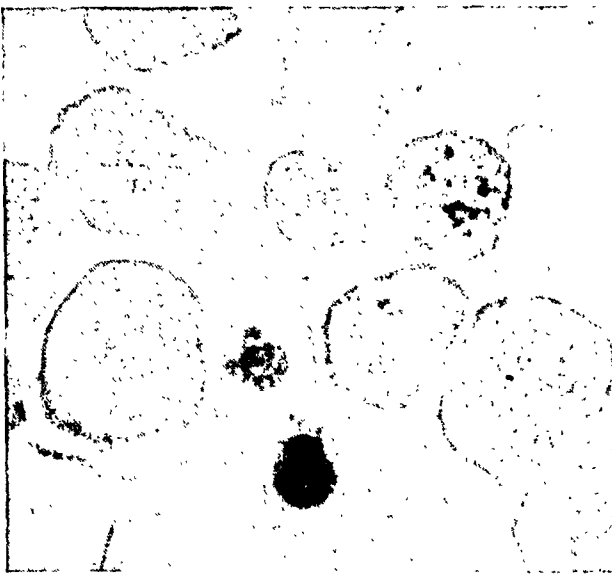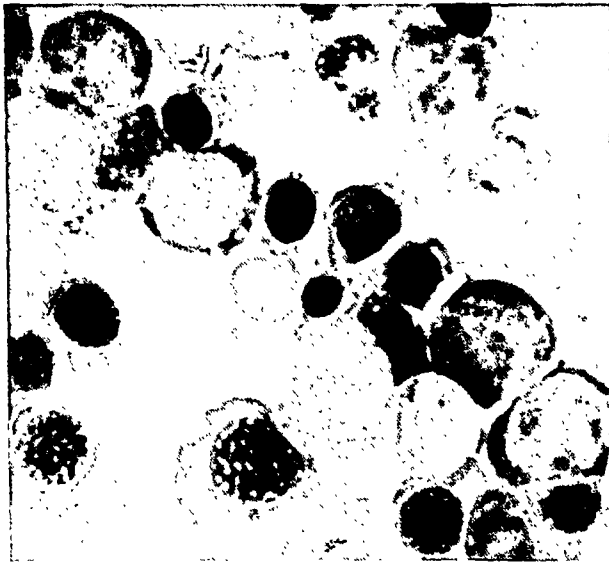
Fig. 1.

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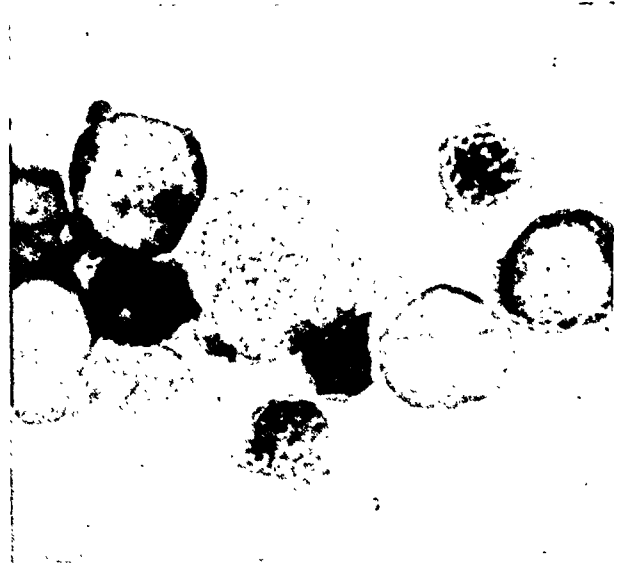
Fig. 4.
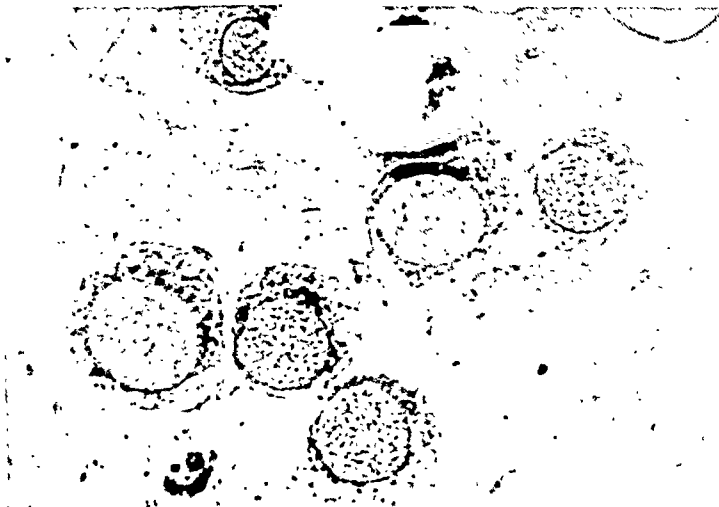
Fig. 5.

FIGS. 1–4.—Bone marrow obtained by sternal puncture biopsy from patients who died of severe refractory anaemia. In Fig. 1 the marrow cells are particularly large and primitive. Fig. 2 shows maturing megaloblasts as well as primitive cells, some of them megaloblasts; also cells with pyknotic nuclei and degenerating cytoplasm. Fig. 4 shows primitive cells and degenerating cells with basophilic cytoplasm.

FIG. 5.—Megaloblasts from the bone marrow of a patient with Addisonian pernicious anaemia, stained by Sudan black by the method of Baker (1945) The mitochondria are chiefly found in the perinuclear zone. Note the deeply stained granulocytes at the top of the figure.

PLATE IV.

polynucleotides in the cytoplasm diminishes moderately in partially differentiated cells but markedly so in the nucleolus. By the time the cytoplasm is polychromatic the concentration of ribonucleic acid has decreased to one-tenth of its maximum value and has completely disappeared in orthochromatic cells. This work, therefore, supports the impression that ribonucleic acid concentration is paralleled by the depth of cytoplasmic basophilia.

Absorption attributable to substances of porphyrin type was not observed in the cytoplasm of the most primitive cells; the earliest cells in which it was first detected were of moderate maturity with polychromatic cytoplasm, at a concentration corresponding to 3 $\mu\mu$g. of haemoglobin per cell. The haemoglobin content increases to 28 $\mu\mu$g. in the most ripened cells with fully pyknotic nuclei, at a concentration of 20 to 25 per cent. The final rise in concentration of haemoglobin to 33 per cent is attributed by Thorell to shrinkage of the cell when it changes from a rounded form as a normoblast to a disc-like erythrocyte.

On the basis of his cytochemical analysis Thorell refers to four phases of activity in the development of the normoblast: (1) a phase of growth where the concentration of ribonucleic acid is maximal, (2) a phase of declining growth where the ribonucleic acids are diminishing and basic cellular proteins like globin are being synthesized, (3) a phase of differentiation when haemoglobin is being formed rapidly, and (4) a phase of declining differentiation.

Although the globin necessary for haemoglobin synthesis is probably formed at an early stage in the life of the erythroblast (through participation of ribose polynucleotides as for protein synthesis generally) the formation of haem compounds and their coupling with globin appears to take place at a later stage, after the ribopolynucleotides have substantially disappeared (see also Shemin and Rittenberg, 1946).

The complexity of the apparatus and methods used by the Caspersson school is a disadvantage. Recently E. M. Jope (1949) and Barer and others (1949) have outlined a simplified technique using the reflecting microscope (Burch, 1947). By this method simultaneous monochromatic cross-sections of the cell structures may be studied (Text Fig. 3). The intracorpuscular haemoglobin of individual erythrocytes produces the same intensity of absorption at the Soret band as was expected from studies of haemoglobin in simple solution.

Formation of haemoglobin.—Knowledge of the mechanism of haemoglobin synthesis is still very incomplete. Duesberg in 1938 published a good review of the position up to that time, and Bur-

mester (1937), in a chemical study of pigment formation in avian erythroblasts, included figures for the iron content of cells at different stages of development. The precursors of the porphyrin ring have for long remained mysterious, but recently Shemin and Rittenberg (1946) have shown that in rat and man glycine labelled with N^{15}-isotope is built into protoporphyrin and ultimately appears in the haemoglobin of the mature erythrocytes. A similar synthesis has been demonstrated *in vitro* when duck erythrocytes and erythrocytes from patients with sickle-cell anaemia were incubated with N^{15}-containing glycine (London and others, 1948). Glycine containing C^{14}-isotope in the α-position has also been used in similar experiments; it is incorporated into both haem and globin moieties of haemoglobin (Altman and others, 1948).

Thorell's work, already referred to, indicates the relatively early diminution in cytoplasmic ribopolynucleotides in polychromatic erythroblasts before the start of haemoglobin formation. H. M. Jope (1948) points out that at this stage the cytoplasmic protein is probably related to the globin of haemoglobin, and that, later, haemoglobin itself is elaborated from this globin, which either forms haem groups on its surface or combines with them after their formation. The cytoplasmic acidophilia of the early polychromatic normoblast is thus due to its content of globin rather than of haemoglobin, which is developed later.

Siderocytes.—An interesting abnormality of the distribution of iron in erythrocytes and their precursors has recently been described. Grüneberg (1941, 1942) observed siderocytes, or erythrocytes with granules giving the Prussian-blue reaction for iron in the blood of normal rat, mouse, and human embryos, and in large numbers in mice with a congenital anaemia. Later they were observed in adult human blood by Doniach and others (1943), and further details have been provided by Pappenheimer and others (1945), Case (1946), McFadzean and Davis (1947), and Dacie and Doniach (1947). Siderotic granules are to be found also in polychromatic and pyknotic normoblasts, usually as a few isolated granules, but sometimes confined to a zone around the nuclear membrane (Dacie and Doniach, 1947).

Siderotic granules of the type described by Grüneberg seem not to be formed in damaged or aged erythrocytes as has been claimed by Case* (1945), but seem rather to be produced at the same time as haemoglobin is elaborated. Possibly a proportion of the iron which normally enters the haem molecule is concentrated in loci in the cytoplasm of the developing normoblast in a relatively free form. It is noteworthy that when many granules

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* Case (1946) has used either α,α'-dipyridyl-thiocyanate or acid potassium ferrocyanide after a preliminary unmasking treatment with ammonium sulphide to demonstrate siderocytes. By these techniques, siderocytes (Case) are present in normal blood and increase in numbers in stored blood. Case believes siderotic granules indicate an ageing cell.

are present the rest of the cytoplasm of the cell stains palely with acid dyes. When stained with basic dyes the siderotic granules appear as basophilic granules (Pappenheimer bodies). The incidence of siderocytes in human pathology has not yet been fully worked out. They are not present in the peripheral blood of normal adults, but constantly appear in small numbers after splenectomy; in certain haemolytic anaemias almost all the corpuscles may be affected.

Abnormal Erythropoiesis

Megaloblastic erythropoiesis. — The term "megaloblast" was used by Ehrlich (1880) to describe the type of nucleated erythroblast found in the bone marrow of patients with pernicious anaemia in relapse. Since that time the terms "megaloblast" and "megaloblastic erythropoiesis" have been widely used in descriptive haematology, but their correct usage and limits, and their significance, have proved to be a controversial problem.

Morphology of the megaloblasts.—Megaloblasts differ from normoblasts in several ways; they are larger cells with increased cytoplasm and nuclear size and at every stage in development they have a different and more open nuclear chromatin pattern, and a tendency in the later types for the cytoplasm to manufacture haemoglobin at stages when the chromatin of the nucleus is still arranged in an open manner. We should like to emphasize several general points before describing the cells themselves. First, that the "megaloblastic" change may be appreciated at all stages in development from the most primitive types to the most mature orthochromatic megaloblasts with pyknosis of the nucleus; secondly, that the degree of abnormality varies greatly between cells of the same apparent age and that all grades of change may be seen between grossly abnormal cells through intermediate types to cells which are almost if not quite normal; and thirdly, that "megaloblasts" are not normally present in the marrow. However, although they are essentially pathological cells, megaloblasts are not a race of cells apart but a type of abnormality* affecting cell growth and maturation, which develops to a greater or lesser extent as the result of deficiencies in growth factors of which the anti-pernicious anaemia factor is an important example.

These points will now be considered in more detail. The earliest megaloblasts, the *promegaloblasts* (Ferrata and Negr iros-Rinaldi, 1914), are larger cells than the pronormoblasts, which are their normal counterparts. In size, they range from 20 to 25 μ in diameter in stained dried films. The cytoplasm is deeply basophilic and relatively abundant, with numerous mitochondria. The chromatin of the nucleus is arranged in a characteristically well defined finely stippled manner, and one or more basophilic nucleoli can be identified. From these basophilic promegaloblasts a series of *ripening megaloblasts* develop. These cells remain larger than normal throughout the ripening process, and the cytoplasmic outline is usually less round than in the normal series. When the cytoplasm attains a polychromatic staining reaction, this is often far in advance of that expected on the basis of nuclear structure, and mitochondria are still numerous. Thus polychromatic megaloblasts with small nucleoli are often observed, and the cytoplasm may even become orthochromatic before pyknosis of the nucleus has been completed. As the cells mature the nuclei become smaller and the chromatin more condensed; nevertheless, the chromatin pattern remains more open than in developing normoblasts. The chromatin takes the form of short strands and small nodes, but there is sufficient "parachromatin" to give an overall mottled effect. The nucleolus-associated chromatin remains finer and more discrete from the rest of the chromatin than in normal development. As the cell matures further the chromatin strands thicken and the nodes enlarge and tend to fuse, but complete homogeneous pyknosis is rarely seen. The nucleus is at first evenly rounded or slightly oval in shape, but often becomes distorted and has an irregular outline before it is fully pyknotic. The mitochondria finally disappear. Abnormal mitosis and nuclear fragments (Howell-Jolly bodies*) are quite frequently seen. The difference between cells developing normally and those with well marked megaloblastic changes is illustrated in Figs. 1–8 and 22–29 of the Coloured Plate and in Text Fig. 1.

Intermediate megaloblasts.—The above description and the illustrations apply to megaloblasts as seen in well stained smears from patients with severe Addisonian pernicious anaemia. The degree of the abnormalities and the extent to which the cells deviate from the normal are closely linked with the severity of the lack of haemopoietic factors. This is illustrated in the photomicrographs of Plate II and the Coloured Plate, and in the Text Fig. 1, in which are shown a series of scale drawings

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* Erythrocyte precursors are not the only developing cells affected by deficiency of the anti-pernicious anaemia factor. Abnormally large and atypical granulocytes are quite as characteristic, but their description is beyond the scope of this article. As in the megaloblasts a characteristic feature is an asynchronism between the growth and development of the nucleus and the cytoplasm.

* Howell-Jolly bodies are small round Feulgen-positive bodies, approximately 0.5 to 2 μ in size. They are derived from nuclear chromatin and may be seen in polychromatic and orthochromatic erythroblasts. It is not certain whether they are produced by nuclear karyorrhexis or are formed from chromosomes, which have been isolated during mitosis. They are found in many types of anaemia and are especially frequent in Addisonian pernicious anaemia, in severe haemolytic anaemias, in idiopathic steatorrhoea (Engel, 1939), and after splenectomy (Singer and others, 1941).

of megaloblasts and a series of normoblasts for comparison. The megaloblasts in the four photomicrographs of marrow films (Plate II, Figs. 1–4) differ not only in size but in the extent to which they deviate from normal cells. These four patients A, B, C, and D had anaemia of varying severity; A and B were the most severe cases, with approximately 1,000,000 erythrocytes per c.mm.; case C was less severe, with 1,800,000 erythrocytes per c.mm.; and case D the least anaemic with 3,400,000 erythrocytes per c.mm. It is evident that not only is the degree of megaloblastic change intimately linked with the seriousness of the lack of haemopoietic factors and that it is hence proportional to the severity of the anaemia in the patient, but that the relative proportion of primitive to maturing megaloblasts is also affected. It is in the severely anaemic patient that the primitive cells are most abundant, and in the mildly anaemic the least abundant. Bilaki-Pasquier (1948) refers to stained marrow containing many primitive megaloblasts as "moelle bleue," and notes that the primitive cells are most numerous when the peripheral erythrocyte count is lowest. See also Leitner (Leitner and others, 1949d).

The nature of the megaloblastic change.—Megaloblasts are produced when there is a deficiency of certain haemopoietic factors, which normally are necessary for effective growth and division and for normal differentiation. These factors may resemble enzymes or coenzymes in their action, and only minute amounts may be required; in the case of the recently purified liver factor (Lester Smith, 1948), it has been calculated that 1 ug. per day is sufficient (Rickes and others, 1948).* The effect of a deficiency of the liver factor is that haemopoiesis becomes disordered and ineffective. The exact details of how this is brought about are still obscure; certainly mitosis is deranged to some extent (Japa, 1945), and may occasionally be multipolar or incomplete, with variation of chromosome numbers in the daughter cells (La Cour, 1944). There seems to be no deficiency in the synthesis of ribonucleic acid as judged by the amount of cytoplasmic and nucleolar basophilia removable by ribonuclease (White, 1947), or by microanalysis (J. N. Davidson and others, 1948)—indeed, the content of both types of nucleic acid in the marrow is increased. The derangement is

a complex one affecting both growth and differentiation and has important effects on cell size, chromatin arrangement, and cytoplasmic ripening, and typically results in an asynchronism between nuclear and cytoplasmic maturation, a point recently emphasized by Bessis (1946, 1948). It has already been mentioned that similar changes may be observed in the developing leucocytes. Thorell (1947b) has studied the distribution of ribose polynucleotides and of haemoglobin in the megaloblasts of marrow from two patients with pernicious anaemia by the micro-spectrophotometric method. He found haemoglobin present in cells that still possessed the same high concentration of cytoplasmic and nucleolar ribose polynucleotides that normally characterize only the more primitive e ythrocyte precursors. Use of the ribonuclease test shows, however, that in those megaloblasts which actually develop to erythrocytes there is a steady decrease of both cytoplasmic and nucleolar ribonucleic acid content. In view of this, it seems possible that Thorell has omitted to analyse the most mature forms of megaloblasts.

The effects on erythropoiesis of a deficiency of the liver principle are mainly twofold: primitive promegaloblasts are present in far larger proportion than are primitive cells in normal marrow, and may total 50 per cent or more of the erythroblasts. In addition there is a variable proportion of maturing abnormal cells (megaloblasts) from which evolve the megalocytes and poikilocytes of the peripheral blood. In addition to primitive cells which definitely have the characters of promegaloblasts, there are also found increased numbers of undifferentiated reticulum cells and transitional stages between the most primitive cells and promegaloblasts and myeloblasts, such cells corresponding to the haemocytoblasts of normal marrows.

As has been mentioned, it is in the most severe cases that the higher proportion of primitive cells is found, and it seems clear that in these patients there is a severe impediment to successful cellular differentiation. It is not so clear what happens to the primitive cells if they fail to mature; in certain of our marrows it is possible to trace stages in shrinkage of cell size and pyknosis of the nucleus, without cytoplasmic ripening, until a small indeterminate remnant results. In the less severely affected marrows the proportion of primitive cells is less, and degenerating forms are inconspicuous; many more maturing megaloblasts are present, and it is obvious that whilst erythropoiesis is abnormal and relatively ineffective, it is at least proceeding. This is borne out by the fact that in these cases the peripheral blood count may remain in equilibrium

* It is outside the scope of this article to consider in detail whether Vitamin B₁₂ is but one of several factors, absence of which will cause megaloblastic change. It is probable that more than one factor is concerned and that the normal chain of processes can be broken at several places. It is generally held that in patients with the sprue syndrome, in certain relatively refractory megaloblastic anaemias, in pernicious anaemia of pregnancy, and in nutritional megalocytic anaemia, liver alone, even in large doses, may not be wholly effective (Wills, 1948). It may be added that pteroylglutamic acid is not exclusively concerned with erythropoiesis, but is a growth factor in a wide biological sense.

for long periods at a moderate level, or at least that it only falls slowly.

The variable nature of megaloblastic marrow.—In the preceding sections on megaloblastic erythropoiesis we have attempted to describe the morphology of megaloblasts and their causation, and have developed the thesis that all grades of change may be recognized between extremely abnormal cells and cells almost indistinguishable from the normal. In this section we shall present further evidence as to the variability of the megaloblasts and of the marrow picture as a whole in patients with Addisonian pernicious anaemia, and will indicate our views as to the abnormal types of erythropoiesis met with in the sprue syndrome and in pernicious anaemia of pregnancy. In the following section we shall compare our own viewpoint with that of other workers.

It is well known that in Addisonian pernicious anaemia the volume of red marrow increases and extends out into the long bones of the skeleton and that there is a diminution or complete loss of the fat cells normally so abundant in the marrow of an adult (see Plate I), and the same is true to a greater or lesser extent in the other allied megalocytic anaemias. Sternal puncture usually provides very cellular material in which marrow particles are readily seen. If differential cell counts are performed an increased erythrogenetic-leucogenetic ratio will be found (Schartum-Hansen, 1937). This is illustrated from our material in Text Fig. 4; there is a general correlation between the severity of the anaemia and the predominance of erythrogenetic cells, but the picture is complicated because of the associated disturbance in leucopoiesis. These marrows are in fact not as one-sidedly erythrogenetic as in chronic haemolytic anaemias with similar degrees of anaemia. In Text Fig. 4 are also shown the results of plotting the degree of anaemia of the patient (a rough indication of the severity of the lack of haemopoietic factors) against the proportion of primitive nucleolated cells with basophilic cytoplasm* amongst the whole population of nucleated erythrogenetic cells. There is an obvious positive correlation between the degree of anaemia and the proportion of primitive cells, and, as has been mentioned before, the megaloblasts become less and less abnormal and more " intermediate " *pari passu* with the falling proportion of primitive cells and reduction in anaemia (Plate II).

Of the thirty-five patients whose data are plotted in Text Fig. 4, nine were suffering from non-Addisonian megalocytic anaemias, and it is particularly interesting to note that the data from these cases fall into line with those obtained from the more numerous patients with typical Addisonian anaemia. In the two severely anaemic patients, one an example of tropical nutritional megalocytic anaemia and the other a patient with pernicious anaemia of pregnancy, there was a high proportion of primitive cells and well marked megaloblastic change; in the less anaemic patients there were fewer primitive cells and the megaloblasts were much more intermediate in type. As far as our experience goes the megaloblastic picture of the non-Addisonian megalocytic anaemias is similar to that of typical untreated Addisonian pernicious anaemia of comparable severity.

Effect on megaloblastic bone marrow of treatment with the liver anti-anaemic principle.—It is well known that the bone marrow of a typical case of Addisonian pernicious anaemia rapidly loses its megaloblastic character and becomes normoblastic if effective doses of liver are given. The exact details and mechanism of this change have been a matter of some controversy (Jones, 1943; Limarzi and Levinson, 1943; Leitner and others, 1949c). It is probable that the ripening megaloblasts complete their transformation into megalocytes if they have lost the capacity for further divisions, but that the primitive nucleolated cells respond to the presence of the liver haemopoietic factor by undergoing normal divisions and differentiating as normoblasts.

The earliest changes are appreciable within twenty-four hours of an injection of liver extract, when basophilic pronormoblasts and normoblasts at an early stage of development may be seen in addition to ripening megaloblasts. During the next forty-eight hours many more ripening polychromatic normoblasts appear and the megaloblasts are less conspicuous, but the latter do not entirely disappear for at least five days, even if the dose of liver produces a maximal response.* If the liver extract is not fully potent a very mixed picture results, "intermediate " megaloblasts replacing to a greater or less extent the expected normoblasts. The completeness or otherwise of the transformation towards normoblastic formation (and the disappearance of abnormal leucocytes) is, naturally, the most delicate index of the adequacy of the liver extract.

A discussion on the megaloblast problem.—The preceding sections have been a presentation of our own point of view, based on our own material. A point of great controversy is the question of " intermediate " forms of megaloblasts; whether they occur at all, what is their significance, and what is their relationship to macronormoblasts. The conception of " intermediate " forms is by no means

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* Reticulum cells and slightly more mature types corresponding to haemocytoblasts which we could not definitely identify as erythrocyte precursors were not counted.

* The change from an inhibited marrow with a high proportion of primitive cells to an actively maturing erythrogenetic one is accompanied by striking reductions in the content of both ribonucleic and desoxyribonucleic acids (J. N. Davidson and others, 1948).

new, but relatively few authors have paid much attention to it. In pre-war Continental literature these forms were referred to under a variety of names, as "macroblasts" by Schartum-Hansen (1937), as "megalo-normoblasts" by Hotz and Rohr (1938) in cases of sprue, and as "intermediate erythroblasts" by Lambin and de Weert (1938, 1939). They have been described by Trowell (1942-3) in tropical dimorphic anaemia and by Zuelzer and others (1947) in megaloblastic anaemia in infancy. Bessis (1946, 1948) in his review of the megaloblast question admits their existence, and Davidson and others (1947) refer to their occurrence in two patients with sprue, and they have been described as "atypical normoblasts" by Cooke and others (1948) in cases of idiopathic steatorrhoea, and as "intermediate erythroblasts" by Innes (1948) in sprue. Our own views have been set out in two previous preliminary communications (Dacie and White, 1947; Dacie, 1948). Other workers have not considered the problem at all or have decided against the existence of intermediate forms (Jones, 1943), and have stressed the striking differences between normoblastic erythropoiesis and gross megaloblastic change rather than the similarities which may be seen between the two series (Israëls, 1939; Leitner and others, 1949f). Most authors, however, including Dameshek and Valentine (1937) and Jones (1938), admit that mixed pictures occur and that a variable proportion of normoblasts may be found in a predominantly megaloblastic marrow.

Not only are we convinced that intermediate forms occur, but we believe that they have a special significance; they appear when the marrow suffers minor deficiencies of haemopoietic principles. They are seen, in our experience, in mild Addisonian anaemia before liver treatment, in more severe examples of Addisonian anaemia that have received suboptimal doses of liver, and in cases of the sprue syndrome and other megalocytic anaemias, where anaemia is relatively mild and response to liver unsatisfactory; presumably in these cases some substance other than the purified liver principle is required, or utilization by the marrow is impaired. We feel that these types of cell deserve a wider recognition and should be referred to as "intermediate megaloblasts" rather than as atypical or intermediate erythroblasts or normoblasts, because their pathogenesis seems to be the same as that of more typical megaloblasts. The difference is merely quantitative and the cells are less definitely abnormal. The question of their relation to macronormoblasts and heteroplastic erythroblasts is discussed in later sections.

Other Types of Abnormal Erythropoiesis

Macronormoblastic erythropoiesis.—The term "macronormoblastic erythropoiesis" has been employed by Jones (1943) to describe the developing erythroblasts in conditions where there is a peripheral macrocytosis not dependent upon a deficiency of the liver principle. This type of blood picture is seen in some chronic haemolytic anaemias (Dameshek and Schwartz, 1940), in liver disease (Bodley Scott, 1939), recovery from haemorrhage (Wintrobe, 1946d; Leitner and others, 1949g), and in patients responding to iron therapy where there has been iron deficiency. It is also found in late foetal life. The term macronormoblast refers to the nucleated precursors of the peripheral macrocytes. Jones, unfortunately, includes an increased proportion of "early forms (pronormoblasts and basophilic normoblasts)" in his conception of a macronormoblastic marrow. To us it seems preferable to use the term solely to describe normoblasts which are larger in size than their normal counterparts of similar age, and not to complicate matters by referring to an increased proportion of earlier types which, even if perfectly normal cells, would increase the average size of the normoblasts. Few authors seem, however, to have demonstrated the abnormal size of "macronormoblasts" by direct measurement. Dameshek and Schwartz (1940) measured the diameters of the erythroblasts in the marrow film of a patient with acute haemolytic anaemia and found the cells to be from 1 to 1.3μ larger than normal cells of the same age group. We have also made some measurements, and contrasted the maturing polychromatic and pyknotic normoblasts in normal subjects with those from patients with macrocytic haemolytic anaemia and iron deficiency anaemia (Text Fig. 5). The results of such measurements as these are, however, open to question, as an increased proportion of more primitive cells produces an apparent macrocytosis, and it is very difficult to measure only cells of strictly comparable age groups. Our figures show, however, that whereas in the five normal marrows 11 to 26 per cent of the cells measure 8μ or less, in the macronormoblastic marrow only 2 per cent of the cells are within this range and in the iron-deficient marrow there are as many as 70 per cent. As these small cells are all mature or almost mature types it seems reasonably certain that real differences exist (see also Plate III, Figs. 1-3).

Do macronormoblasts differ from normoblasts in any respect other than size? Their nuclear structure resembles that of normoblasts and thus differs from that of intermediate megaloblasts. Israëls (1941) has stated that premature haemoglo-

binization of the cytoplasm of erythroblasts (presumably macronormoblasts) often occurs in hyperplastic marrows with normal maturation. This change seems to us to be a slight one and unreliable as a diagnostic aid. We often experience some difficulty in the recognition of individual macronormoblasts, but believe that this is only to be expected because both macronormoblasts and intermediate megaloblasts merge into the normal. The general trend of both abnormal types is, however, quite distinctive if the abnormalities are marked.

Little is known as to why macronormoblasts develop or about the mechanism of their formation. In most instances they are found when haemopoiesis is proceeding rapidly and when there is a raised proportion of reticulocytes in the peripheral blood. Possibly in a hyperplastic marrow unripened cells are forced into the blood stream because the active growth of the marrow as a whole outstrips the maturation of individual cells. Abnormal size and occasionally the persistence of a nucleus, as well as cytoplasmic basophilia, may thus all be signs of immaturity. An alternative hypothesis is that large ripened normoblasts are produced as the result of a diminished number of cell divisions through which they have been derived from the pronormoblasts. The differences between the abnormal and normal are relatively slight, however, and it seems unlikely that a stimulated, actively erythrogenetic marrow would respond to the demand for more erythrocytes by reducing the number of cell divisions.

Another hypothetical possibility is that there is normally some humoral mechanism which controls the development and maturation of the erythroblasts, similar to that described as controlling the maturation of reticulocytes (Jacobsen, 1947), and that in conditions of rapid erythropoiesis there may be a relative deficiency of ripening factors. At the moment all that can be said is that none of these hypotheses has been proved.

Erythropoiesis in iron deficiency (Micronormoblastic erythropoiesis).—The effect of iron deficiency on the erythrocytes of the peripheral blood is well recognized, but rather less is known about the morphology of the erythrocyte precursors in the marrow, and as to how deficiency of iron results in disordered erythropoiesis. Bodley Scott (1939) has reviewed the early literature and described his own findings in twenty-three patients. He found an increased cellularity of the marrow and an increased proportion of erythrogenetic cells roughly parallel to the severity of the anaemia. He described the predominant cells as polychromatic normoblasts " with an irregular and jagged cell outline and only a small rim of slate-grey cytoplasm around the pyknotic nucleus." Since Bodley Scott's paper the disordered erythropoiesis of iron deficiency has received wider recognition (Wintrobe, 1946e; Whitby
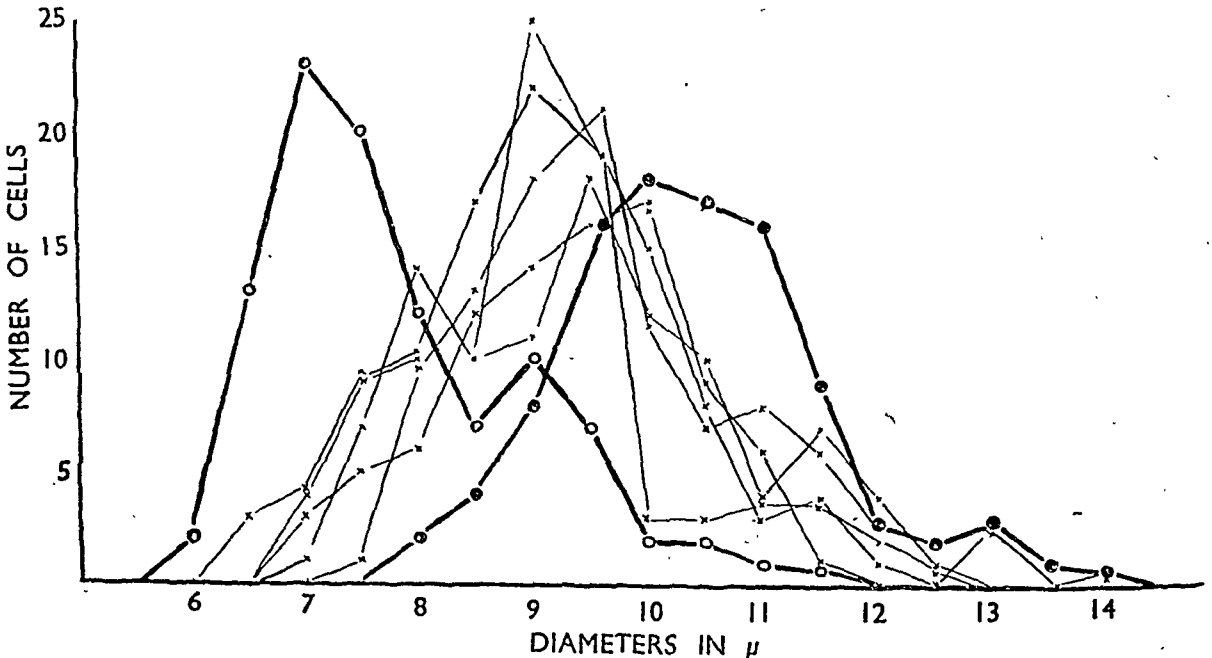
Text Fig. 5.—Diameter distribution curves of 100 polychromatic and pyknotic normoblasts, measured in sternal marrow films from five normal subjects x———x, a patient with a macrocytic haemolytic anaemia ●———●, and from a patient with an iron deficiency anaemia o———o. (For interpretation, see text.)

and Britton, 1946), and might well be referred to as " micronormoblastic " in type (Plate III, Fig. 2, and Text Fig. 1). The haemocytoblasts and pro-erythroblasts in iron deficiency seem normal in size and appearance and are usually present in a normal or slightly increased relative proportion, but there is usually an increase in the numbers of basophilic normoblasts (Leitner and others,1949h). Our own observations and measurements (Text Fig. 5) confirm the small size of the more mature normoblasts and are in agreement with Bodley Scott's description. Cytoplasmic ripening seems to lag behind nuclear condensation, and fully orthochromatic cells are not seen.

The presence of a hyperplastic erythrogenetic marrow in association with peripheral anaemia has led to the view that there is an arrest of maturation similar to that found in Addisonian pernicious anaemia. However, there is little obvious morphological evidence for this; not only are more normoblasts present than in the normal, but a high proportion seem to be maturing. It might be thought that the malformed hypochromic erythrocytes and poikilocytes are unduly sensitive to the normal process of wear and ' tear and that this contributes significantly to the anaemia, which is in effect partly haemolytic* in type, and that the presence of so many normoblasts indicated a rapid ε cell production. However, the level of reticulocytes in the peripheral blood in a case of untreated anaemia due to iron deficiency is rarely above 2 or 3 per cent, and the serum bilirubin is low. The fact that effective iron therapy leads to an outpouring of reticulocytes is in favour of the previous existence of some sort of retardation of development. It is possible that the effect of a lack of a sufficient level of plasma iron is a failure of the last stages of normoblast differentiation in addition to inadequate synthesis of haem, and that the ragged-bordered polychromatic but almost pyknotic normoblasts are dying cells, the most severely affected of which never become erythrocytes at all. The reduced size of these normoblasts is presumably directly due to the reduced synthesis of haemoglobin, resulting in decreased globin formations from its precursors, and hence a small cytoplasmic mass.

Erythropoiesis in refractory anaemia.—The refractory anaemias (Bomford and Rhoads, 1941) are dyshaemopoietic anaemias often referred to by the descriptive titles aplastic, hypoplastic, or pseudoaplastic, or as panmyelophthisic anaemias. For convenience we are also including " refractory

megaloblastic " and achrestic anaemias. The whole group is probably heterogeneous, but has in common a resistance, more or less complete, to any treatment now known. It is because we do not think that the descriptive titles mentioned above necessarily indicate distinct entities that we hesitate to discard the more general term " refractory anaemia." As a rule leucopenia or thrombocytopenia accompanies the anaemia, which is normocytic or macrocytic in type.

The only aspect of this group that we are now considering is the cytology of erythropoiesis. There have been several important studies in recent years. Israëls and Wilkinson (1940) described as suffering from " achrestic anaemia " six patients in whom the biopsied marrow was found to be megaloblastic or mixed megaloblastic and normoblastic. Bomford and Rhoads (1941), basing their account on sectioned biopsy or autopsy material, reported the marrow to be partly mature and cellular, or hypoplastic, or immature and cellular; and Davidson and others (1943) in a smaller series of cases reported the marrow, as studied by sternal puncture, to be hypocellular and normoblastic, or hypercellular and megaloblastic. Other patients in whom the marrow appearances greatly varied are described by Leitner and others (1949i).

In our own material we have observed great variation in the type of erythropoiesis. This variability is illustrated by the details of the following seven patients. In the first case there was a primitive marrow containing numerous haemocytoblasts and increased numbers of reticulum cells, with but few ripening cells, mostly intermediate megaloblasts, and a normocytic peripheral picture (Plate III, Fig. 4). In the second patient the marrow was extremely primitive, with some differentiation to typical megaloblasts, and a megalocytic peripheral blood picture (Plate III, Fig. 1). In the third patient the marrow was hyperplastic; primitive cells were present in moderately large numbers, and there was a moderate proportion of differentiating cells, some being megaloblasts. In addition, degenerating cells with pyknosis of the nuclei but without cytoplasmic ripening were conspicuous. The peripheral blood picture was of a megalocytic anaemia (Plate III, Fig. 3). In the fourth patient the marrow although hypoplastic at autopsy, was shown during life by sternal puncture to be primitive, but to contain differentiating cells definitely megaloblastic in type (Plate III, Fig. 2). In the marrow of the fifth patient found to be hypoplastic at autopsy, there was in life, on the other hand, very little evidence of a megaloblastic change; most of the nucleated erythroblasts were normoblasts, a few were intermediate megaloblasts. The sixth patient, less anaemic than the foregoing, had a moderately hyperplastic and partly primitive marrow; once again the differentiating cells were mostly megaloblasts of intermediate type. In the seventh patient the marrow at biopsy appeared to be hyperplastic but not

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* It is possible that this mechanism may contribute to the anaemia of Mediterranean anaemia (Cooley's anaemia). The hypochromic erythrocytes are extremely deformed, and there is evidence suggesting increased haemolysis (Dameshek, 1943).

primitive; erythropoiesis was normoblastic and normal except for some macronormoblasts with almost pyknotic but distorted nuclei. Anaemia was, however, severe and slightly macrocytic in type.

It is difficult to understand the mechanism of anaemia in those patients who have a hyperplastic and differentiating marrow (pseudo-aplastic anaemia). Presumably in these cases the final maturation of the normoblasts is unusually slow or the ripe cells may die before reaching the circulation. Possibly also, in some cases, hyperplastic areas alternate with areas of hypoplasia. In the patients whose marrows are predominately primitive in type, the mechanism of the failure to deliver adequate numbers of erythrocytes into the peripheral circulation is probably similar to that which operates in Addisonian pernicious anaemia. The primitive cells fail, to a greater or less extent, to complete their differentiation, and may be seen to undergo pyknosis of the nucleus and to degenerate without differentiating at all. Of the maturing cells a variable proportion have characters more or less typical of megaloblasts. As with our own series of patients with typical Addisonian anaemia, the more seriously anaemic the patient the more inhibited and primitive the marrow is likely to be.

Where aplasia seems to follow a proliferating marrow of primitive type, this change may well be due to an intensification of the factors causing the anaemia, whether they be " toxins," lack of growth factors, or the presence of a growth antagonist. This change from hyperplasia to hypoplasia of the marrow was observed by Bomford and Rhoads (1941) in some of their patients. Marrow cellularity, diminishing as the disease progressed, seemed to be a characteristic feature of the group of patients whose marrows were shown to be hypercellular and " partly mature " at biopsy; in five patients, however, the change was from a " partly mature " marrow to an immature one.

Erythropoiesis in haemolytic anaemia.—Increased haemolysis leads almost invariably to increased erythropoiesis,* and, in marrow biopsy smears of patients with an active haemolytic anaemia, erythropoietic cells are usually extremely numerous. The type of erythropoiesis has almost always been described as normoblastic (Leitner and others, 1949j). Turnbull (1936c) has, however, identified megaloblasts in addition to normoblasts in postmortem sections of marrows from several patients with acholuric jaundice who died of haemolytic crises, and he has suggested that in these cases

there may have been a deficiency of haemopoietic factors. We have not yet encountered typical megaloblasts in marrow smears from our own patients; the normoblasts, however, tend to be a little larger than normal and may thus be described as macronormoblasts. This increase in size is most easy to recognize in the most mature cells. In addition to this we have noted, in patients where haemolysis is severe, that complete nuclear pyknosis is infrequent and that the nucleus of the almost mature cells with polychromatic cytoplasm may be slightly more " open " than in normal normoblasts, in relation to the acidophilia of the cytoplasm. Occasionally, the most mature nuclei may be irregular in shape. The whole picture is, however, unmistakably normoblastic rather than megaloblastic.

Erythropoiesis in leukaemia and allied disorders.—The association of anaemia with leukaemia is almost invariable, except in the earliest stages. In the peripheral blood the presence of undue anisocytosis and poikilocytosis and of erythroblasts suggests that erythropoiesis itself is disordered. Occasionally the disorder of erythropoiesis is dominant and the leucocyte abnormalities subsidiary or even subsequent; such cases, in which a severe macrocytic anaemia with evidence of abnormal erythropoiesis precedes the development of indubitable leukaemia, have been referred to as " leukanaemia " (Leube, 1900; Forkner, 1938; Foy and others, 1946). In addition to this variety of leukaemia there are patients in whom the *proliferative* process predominately and persistently affects erythrogenetic cells. This rare disorder was first described by di Guglielmo* as acute erythraemia or erythraemic myelosis. " Leukanaemia " seems to occupy an intermediate position between erythraemia and leukaemia.

In myelogenous, lymphogenous, and monocytic leukaemia, erythropoiesis is predominately normoblastic, but a small proportion of cells simulating intermediate megaloblasts may be found; occasionally, and often towards the termination of the illness, these cells are present in higher proportions. " Megaloblasts " have been reported more frequently in cases of leukanaemia (Penati, 1937; Foy and others, 1946; Collins and Rose, 1948). In acute erythraemia, di Guglielmo has described as " paraerythroblasts " the atypical cells which are frequently encountered, and whose origin in some cases may be traced from the proliferating reticulum cells (haemohistioblasts). Atypical mitoses and irregular and multilobed nuclei are not infrequently

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* Owren (1948) has demonstrated that *hypoplasia* of the erythropoietic marrow cells may be a cause of severe crises in congenital haemolytic anaemia; lack of maturation of primitive erythroblasts may also be a cause (Dameshek and Bloom, 1948).

* In di Guglielmo's (1946) review in French there is a full bibliography of his earlier papers.

seen in more mature forms of these cells, and there may also be unusually early acidophilia of the cytoplasm. In general, however, the type of erythropoiesis has been described as normoblastic, although amongst the "paraerythroblasts" there are some cells which closely resemble megaloblasts; these have been called "megaloblastiform" cells by Heilmeyer and Schoener (1941), and this term has been used by di Guglielmo himself and by Bessis (1948). Presumably also the patient described by Downey (1938b) as suffering from leukaemic reticulo-endotheliosis, in which megaloblast-like cells were observed to be developing heterotopically from reticulum cells, and the first patient described in Schleicher's (1944b) paper represent examples of a further rare variant of the leukaemia-erythraemia group.

We have observed cells rather similar to intermediate megaloblasts in leukaemia (Plate III, Fig. 5) and in erythraemia. These cells are rather larger than normal for their apparent maturity, and the acidophilia of the cytoplasm is in advance of the form of the nucleus. In general, we believe that this change towards a "megaloblastiform" type of erythropoiesis cannot be influenced by liver or folic acid therapy. In all probability it indicates more than a deprivation of growth factors by the rapid growth of leukaemic tissues, and is rather a reflection of the disordered growth caused by the "leukaemic" stimulus itself affecting erythropoiesis, and resulting in changes in morphology not unlike those caused by minor deficiencies in the liver haemopoietic principle (see also Schwarz, 1946).

Erythropoiesis in carcinomatosis of the bone marrow.—The leuco-erythroblastic anaemia of carcinomatosis of the bone marrow has been well described by Vaughan (1936a and b) and Turnbull (1936d). They have reported the presence of megaloblasts of Ehrlich with premature ripening of the cytoplasm in the bone marrow and in the peripheral blood. Both Vaughan and Turnbull remark, however, on the small size of the megaloblasts; in Turnbull's description of the marrow findings "most are relatively small megaloblasts with scanty cytoplasm and some are not appreciably larger than normoblasts. . . . Erythropoiesis is, therefore, partly megaloblastic, partly normoblastic, the normoblastic usually predominating." Bessis (1946) has also referred to intermediate types of "erythroblasts" when the bone marrow is invaded by metastatic carcinoma. In our view it is uncertain whether these abnormal cells should be considered as macronormoblasts or megaloblasts; further study is required. In a recent case erythropoiesis was predominantly normoblastic, but isolated cells

resembling intermediate megaloblasts could be found (Plate III, Fig. 6). Possibly both types of reaction occur. The cause of these changes is obscure. Vaughan concluded that the growing tumour cells interfered with the metabolism of the erythrogenetic cells; this may well be the case.

Cellular gigantism in human erythropoiesis.—The occurrence of cellular gigantism has recently been reviewed by Schwarz (1946) and by Berman (1947). Binucleated "erythroblasts" may be found in both normal and abnormal bone marrows and they probably arise by endomitosis.* Schwarz (1946) discusses at length the abnormalities in mitosis which give rise to these multinucleated cells and describes the very large erythrocytes (gigantocytes), 18–25 μ in size, to which they give rise. He stresses the fact that no intermediary links are found between normal cells and gigantocytes and their multinucleated precursors. He does not consider that these giant cells are ever produced by amitosis. Berman found in eight normal subjects from 1 to 5.1 binucleated cells per 1,000 normal erythroblasts. Multinucleated erythroblasts produced by multipolar mitosis are, however, not found in health; not one example was seen by Berman out of 53,167 erythroblasts examined in his eight normal marrows. In disease, however, they occur not infrequently. Limarzi and Levinson (1943) have described three types of multinucleated erythroblasts: the first is produced as the result of multipolar mitosis without cytoplasmic separation; the second arises by folding and lobulation of the nucleus and finally by separation of the nuclear fragments; and the third by complete or incomplete amitosis —a mechanism which has been questioned (Schwarz). The resulting giant cells are well illustrated by Limarzi and Levinson (1943), Schleicher (1944b), Schwarz (1946), and Berman (1947).

Multinucleated erythroblasts have been observed in a variety of blood disorders; in Berman's series, in cases of leukaemia, lymphoblastoma, pernicious anaemia, congenital haemolytic anaemia, thrombocytopenic purpura, and liver disease. Schleicher (1944b) reported large multinucleated cells in a patient with reticulum-celled sarcoma (reticuloendotheliosis) of the bone marrow and in pernicious anaemia. Limarzi and Levinson's case was considered to be an example of erythroblastoma, and we have recently encountered a good example in a patient with pernicious anaemia of pregnancy (Plate III, Fig. 4).

Berman has stressed the fact that these giant cells are not specific for any particular disease process, that the process may be reversible, as in pernicious anaemia, and that the nuclear and cytoplasmic patterns conform to the type of the accompanying uninucleated cells. Thus multinucleated normoblasts or megaloblasts may be found, or giant dysplastic types with some of the characters of megaloblasts, as in Limarzi and Levinson's case.

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* Endomitosis is complete nuclear division without division of the cytoplasm.

Although, therefore, the exact cause of the aberrant nuclear divisions remains obscure, it is clear that these processes do not necessarily indicate a malignant (" leukaemic ") process. The position seems to be very similar to that for the occurrence of giant or multinucleated cells in other sites throughout the body. In the marrow they are most frequent during active erythropoiesis, as they are in other tissues during rapid growth phases (for example, in the testis of the normal mouse—Howard, 1948).

Abnormalities of mature erythrocytes.—An almost constant feature of anaemia is an increased variability in the size (anisocytosis) of the mature erythrocytes over and above the normal cell to cell variation. In addition, in most anaemias, corpuscles of abnormal shape and not strictly round in contour (poikilocytes) are found. The normal variation in cell diameters has been well worked out by Price-Jones (1933), who has shown that it conforms to a normal biological distribution, and similar variations in volume and thickness, etc., also probably exist. The cause of the exaggerated variation in anaemia is so far unsettled; anisocytosis and poikilocytosis are marked in dyshaemopoietic anaemias, particularly in Addisonian pernicious anaemia in severe relapse, in Mediterranean anaemia, and in some examples of leukaemia and myelosclerosis, and to a lesser extent in iron deficiency anaemia. In haemolytic anaemias and other anaemias with active regeneration, poikilocytosis is much less obvious, and variation in size is partly due to the large size of the polychromatic corpuscles. Spherocytic microcytes* may also be present and may add to the range of cell diameters. Poikilocytosis may thus be taken to indicate disordered erythropoiesis, and this is probably also true of marked anisocytosis, although in this case the production of macrocytes may be due to rapid rather than to abnormal formation.

To authors such as Plum (1947) and Boström (1948), who believe that erythrocytes are produced by budding from the cytoplasm of erythroblasts, the problem of anisocytosis and poikilocytosis presents no difficulty. The budding process is held to be deranged so that buds of different sizes are formed. Boström considers that poikilocytes are produced by cytoplasmic budding taking place through pathologically thickened walls of medullary sinusoids, and schematically illustrates this. However, as already mentioned, these unusual conceptions are not easily acceptable. Nevertheless, it is possible that the small poikilocytes of pernicious anaemia are cytoplasmic fragments (schistocytes: see Rous, 1928), but whether they are formed in the marrow (Habelmann, 1940) or represent fragments broken off larger cells in the peripheral blood stream is uncertain. In general we feel that the solution of the problem of the formation of poikilocytes depends on an understanding of the forces

which convert the cytoplasm* surrounding the nucleus of the normoblast into the disc-like form of the reticulocyte. It is easy to imagine that when erythropoiesis is abnormal this process is deranged and that imperfect erythrocytes result. Anisocytosis is likely to be a reflection of variations in the size of the normoblasts of the same apparent age. That this variation exists is illustrated in the normoblast diameter distribution curves (Text Fig. 5) and in Plate III, Figs. 1–3. Very large erythrocytes (gigantocytes) are occasionally encountered. They arise from multinucleated erythroblasts produced by abnormal mitosis (Schwarz, 1946).

Conclusions

In this review we have attempted a survey of erythropoiesis in man. The subject has many ramifications and a vast literature. While mentioning, therefore, some publications of historical interest, we have mostly referred to modern work. As our aim has been to present as wide a picture of erythropoiesis as possible, we have not confined ourselves to morphology and cytogenesis; we have included sections on the growth and differentiation of the erythropoietic tissue and a discussion of the cytochemical aspects of erythropoiesis, for not only is the mature erythrocyte a perfect example of the differentiation of a cell for a highly specialized function, but the sequence of changes accompanying development affords good examples of the processes of tissue growth as a whole.

The general pattern of erythropoiesis has been described in health and in disease. In health, a bone marrow biopsy gives a momentary but still picture of what is really a carefully regulated dynamic process, but even here much of the mechanism of normal erythropoiesis is ill-understood. In disease, the pattern of erythropoiesis may be greatly altered; accelerated, abortive, and aberrant formation may all be seen, sometimes in combination. Here, too, the changes are dynamic. All grades of change may be seen between the most abnormal bone marrows and a marrow scarcely distinguishable from the normal.

Bone marrow biopsy provides an almost unique opportunity for the examination of living human tissue. It is an indispensable adjunct to the proper understanding of the diseases of the blood.

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* " Microspherocytosis " is a change which takes place after the erythrocytes have been delivered from the bone marrow. The marrow normoblasts are not abnormal; neither, as a rule, are the reticulocytes. The same is true of elliptical corpuscles (see Leitner and others, 1949k) and of sickle cells (Wintrobe, 1946f). The antithesis of spherocytosis target-cell formation seems, in Mediterranean anaemia at least, to depend upon an abnormality of formation.

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* Pronormoblasts and ripening normoblasts are probably almost spherical cells. As the nucleus shrinks and the cytoplasm becomes more abundant relatively, the cell is roughly elliptical in vertical cross-section.

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ESTIMATION OF PROTHROMBIN IN DICOUMARIN THERAPY

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The concept of prothrombin as the specific precursor of thrombin dates from the so-called classical theory of blood coagulation put forward by Schmidt (1895) and Morawitz (1905). This theory, which considers that four factors, thromboplastin, prothrombin, calcium, and fibrinogen, are concerned in the formation of fibrin, has been the basis of most modern work. The theory was of more academic than practical importance until the discovery of vitamin K and the use of dicoumarin in treatment of thrombosis, both of which required the quantitative estimation of prothrombin. The available methods are all based on the assumption that the classical theory is fundamentally sound.

Of the methods of prothrombin estimation that have been developed, none can measure prothrombin directly and therefore infer its concentration from observations on thrombin. The one-stage technique of Quick (1942) has been the most commonly used because it is simple, easily performed, requires little in the way of special reagents, and gives results which are in general agreement with clinical experience. The method consists of adding an optimum amount of brain thromboplastin and calcium to oxalated plasma, under which conditions the clotting time of the mixture is assumed to be proportional to its prothrombin concentration. The basis of this supposed relationship is the literal acceptance of the classical theory. If three of the four variables are controlled, then variations in the fourth must determine any alteration in the rate of fibrin production, which is in turn indicated by a change in the clotting time of the mixture. In practice thromboplastin and calcium are controlled, if possible at or near their optimum concentrations, and fibrinogen variations found in the ordinary clinical material are usually not of sufficient magnitude to influence the result. The remaining variable is therefore prothrombin.

The method is essentially dynamic in principle, depending entirely on the rate of thrombin generation and not on the amount of thrombin that may finally be produced. It depends also on the assumption that there are no uncontrolled factors other than prothrombin that might modify this rate of thrombin generation. It is now known, of course, that this is not the case. Not only may the reaction time of fibrinogen vary, but accelerators and depressors of thrombin generation are present in normal plasma, and variation in these accelerators and depressors in pathological conditions may alter the results of this test. Quick (1942) himself has been the first to recognize this. The practical implications of the fallacious nature of this method are difficult to assess at present, but no alternative to it is available for routine use. Though theoretically the two-stage method appears to be on much firmer ground, since it seeks to estimate the total amount of thrombin generated, it is too complex for ordinary routine use. There are, moreover, many indications that its results may be equally fallacious.

In dicoumarin therapy one component of prothrombin (prothrombin B of Quick, 1947) is deliberately lowered, and the responsibility for a reduction to haemorrhagic levels rests largely with the pathologist. A reliable method of prothrombin determination is therefore essential. There has recently been much discussion as to which of the modifications of the one-stage technique give the best results (Marsh, 1947, 1948 : James, 1948 : Cleland, 1947 : Pivawar, 1947 : Lempert, 1948 : Canti and Robertson, 1948).

Quick's Technique using Brain Thromboplastin in Dicoumarin Therapy

Quick's technique using brain thromboplastin has been used to follow the effects of dicoumarin

administration in numerous animal experiments and human cases (Bingham and others, 1941 ; Butsch and Stewart, 1942 ; Meyer and others, 1942 ; Shapiro and others, 1943 ; Barker and others, 1945 ; Bingham and others, 1943 ; Gefter and others, 1944 ; Barker and others, 1943 ; Jaques and Dunlop, 1945a ; Quick, 1945 ; Cotlove and Vorzimer, 1946 ; Allen, 1947 ; Glueck and others, 1948 ; etc.). In dogs single doses of 5 mg. per kilo can be relied upon to cause a fall in prothrombin to 10 per cent in 24 to 48 hours. In Allen's (1947) series of 1,686 cases a dose of 300 mg. was given on the first day and 200 mg. on the second (equivalent to 5 to 8 mg. per kilo) ; there was a fall in prothrombin within 24 to 72 hours. Other authors recommend a similar dosage and their cases followed the same general course. In a series of a thousand cases Barker and others (1945) claimed that haemorrhage was uncommon when the level of prothrombin was above 10 per cent (indicated by a lengthening of coagulation time from 19 to 60 seconds). A similar " danger level " has also been recorded in vitamin K deficiency. There is, therefore, evidence that prothrombin estimation by this method is in general agreement with the clinical manifestations of hypoprothrombinaemia.

One-stage Technique using Russell's Viper Venom as Thromboplastin

Modifications of the technique in use in this country involve the replacement of brain thromboplastin with Russell's viper venom (Fullerton, 1940 ; Page and Russell, 1941 ; Page and others, 1941–2a and b ; Page and de Beer, 1942–3 ; Page and de Beer, 1943 ; Shapiro, Sherwin, Redish, and Campbell, 1942) or Russell's viper venom and lecithin (Witts and Hobson, 1940 and 1942 ; Hobson and Witts, 1941). It has been recognized for many years that certain snake venoms are powerful coagulants, and Martin (1894) was one of the first to investigate their action. Some, such as the venom of *Echis carinatus* and *Notechis scutatus*, are thrombin-like in nature as they clot oxalated blood. Lamb (1903) observed that Russell's viper venom was almost devoid of this thrombin-like effect, and the immensely powerful thromboplastin-like action of the venom was largely overlooked until Macfarlane and Barnett (1934) reinvestigated its possibilities, in their search for a local haemostatic effective in haemophilia. It was found that this venom was capable of accelerating the coagulation of haemophilic blood in dilutions as low as 1 in 10^{12} or more, but was incapable of clotting oxalated or citrated blood

or fibrinogen solutions. It was thought, therefore, to act as a thromboplastin, a view in accord with Mellanby's (1909) conclusions as to reactions of other viper venoms.

Following its use as a local haemostatic, Russell's viper venom was made available as a commercial product under a number of trade names. It was obtainable as a dry, sterile, and very stable preparation of tested potency in sealed ampoules. Such preparations have obvious advantages over the more usual brain thromboplastin employed in Quick's original technique, as this is often difficult to obtain, not very stable, and of widely varying potency. These advantages were recognized by Fullerton (1940), who suggested the use of venom as substitute for brain thromboplastin. The use of Russell's viper venom as a substitute for brain is only justified, however, if it is shown to behave in all significant respects like the natural thromboplastin. Several observations have suggested that this is not the case. Trevan and Macfarlane (1936) found that the action of the venom was greatly potentiated by tissue extracts and by use of lecithin. Macfarlane (1938) observed that plasma deprived of platelets by high-speed centrifuging was only slowly clotted by venom, while Macfarlane and others (1941) showed that the removal of lipoid from the plasma inhibited its coagulation by venom, but this could be restored by the addition of various lipoid substances including lecithin. It appeared, therefore, that some lipoid co-factor was essential to the action of the venom, and it was thought with Leathes and Mellanby (1939) that natural thromboplastin might consist of an enzyme and lipoid co-factor analogous to Russell's viper venom and lecithin. Theoretically, therefore, the venom corresponds only to a part of the complete thromboplastin. In 1940 Witts and Hobson suggested the use of venom and lecithin mixture as a thromboplastin for the one-stage method as being more nearly related to brain and providing a more rapid conversion of prothrombin. Such a mixture shortens the prothrombin time to 6 or 7 seconds.

There are thus obvious differences in action between venom and natural thromboplastin, and even the supposition that the venom corresponds to a part of such a thromboplastin is unproved. It is therefore surprising that its use as a substitute for brain with or without the addition of lecithin has become widespread, particularly in England, without an adequate series of determinations in parallel with brain. Fullerton (1940) compared Russell's viper venom and brain in 43 cases, but gives no figures in his paper. Page and Russell

(1941) made a similar study in 71 cases, but few had any gross prothrombin deficiency and the coagulation times as presented cannot be assessed in relative percentage of prothrombin. Witts and Hobson (1940) tested the venom and lecithin method in 40 cases, but only two of these had prolonged prothrombin times, and Page and others (1941–2b) noted that the method failed to record minor degrees of prothrombin deficiency, evident when the venom alone was used.

With the advent of dicoumarin therapy it became more obvious that venom and lecithin did not give results comparable to those obtained with brain. Witts (1942) estimated the prothrombin time in a dog following a large dose of dicoumarin and found that both with venom alone and with venom and lecithin the apparent decrease in prothrombin was less than was the case if brain was used for the estimations, an observation supported by de Beer (1947) for venom alone. Wright and Prandoni (1942), using venom for prothrombin determinations, found it necessary to give much larger doses of dicoumarin to achieve a reduction in prothrombin than were advised by other workers using brain for prothrombin estimation. In one case as much as 8,000 mg. were given. Moreover, the apparent fall in prothrombin was delayed from eight to ten days and eight serious haemorrhages occurred in the first 20 cases treated. Shapiro, Sherwin, and Gordimer (1942), and Shapiro and Sherwin (1943) made a similar study, but in only two cases were the actual prothrombin times recorded. In one of these, 700 mg. were given in the first two days and there was a fall in prothrombin to 30 per cent (as estimated from our dilution curves) on the fifth day. In the second there was a fall to 15 per cent following 100 mg. of dicoumarin for six days.

The scanty evidence from the literature therefore suggests that modifications of Quick's technique using Russell's viper venom with or without lecithin may give a less sensitive estimation of prothrombin deficiency. This evidence is supported by the two cases under treatment with dicoumarin recorded by Lempert (1948). In the first of these Quick's technique gave 10 per cent prothrombin, where by Fullerton's technique the level was 35 per cent. In the second case, Fullerton's technique showed 25 per cent prothrombin and Quick's technique 10 per cent. A series of cases recently treated in this hospital supports the findings of Lempert, and suggests that Witts and Hobson's technique may give a dangerous impression of security in following the course of dicoumarin therapy.

Technique

(a) QUICK'S METHOD

1. Collection of blood.—Venous blood is collected in a dry syringe and added to 2 per cent potassium oxalate solution in the proportion of nine parts of blood to one part of oxalate. The mixture is inverted several times and the plasma removed after centrifuging at 1,500 r.p.m. for 15 minutes. The plasma should be kept in the refrigerator and used within 6 hours of collection because the coagulation time of pathological plasmas may alter rapidly on storage.

2. Preparation of thromboplastin.—Fresh human brain is collected from the post-mortem room, all superficial vessels and meninges are removed, and the substance is macerated with acetone in a mortar. The acetone is replaced several times and the granular powder dried on a suction filter. When completely free from acetone the powder is kept in bottles at room temperature. This material can be stored for three months without deterioration.

3. Preparation of thromboplastin for use.—0.5 g. of the dried powder are suspended in 5 ml. of 0.85 per cent saline and the mixture is incubated at 37° C. for 15 minutes. The suspension is centrifuged for 5 minutes at 1,500 r.p.m. and the supernatant emulsion is diluted 1 in 10 with 0.85 per cent saline. The solution should be made up freshly as required, and this concentration was usually found to be the optimum for levels of prothrombin between 5 and 100 per cent (see below).

4. Calcium chloride solution.—2.875 g. of anhydrous calcium chloride are dissolved in 1,000 ml. of distilled water to make a 0.025 molar solution.

5. Performance of the test.—0.1 ml. of undiluted normal plasma is added to 0.1 ml. of brain emulsion and the mixture is warmed to 37° C. in a water bath. 0.1 ml. of 0.025 M calcium chloride is then added rapidly from a graduated Pasteur pipette and the coagulation time is recorded from the time of addition of the calcium. The test should be made in triplicate and the mean of three readings recorded. The same procedure is repeated with the abnormal plasma. If the normal plasma gives a clotting time of more than 30 seconds the technique is unsatisfactory.

6. The optimum concentration of brain thromboplastin and calcium

(a) Brain thromboplastin.—Aggeler and Lucia (1938) have shown that prolongation of the coagulation time occurred with both high and low concentrations of brain emulsion. With normal undiluted plasma the range within which a minimum coagulation time occurred was wide, but with pathological or diluted plasmas the optimum range was limited. Similar results were obtained in this laboratory. The optimum concentration usually corresponded to the dilution mentioned

above, but different batches of brain vary slightly. The optimum concentration may be determined as follows: 0.5 g. of dried brain are suspended in 5 ml. of saline and the supernatant emulsion removed as described above. This concentration is called 100 per cent, and from this a number of dilutions are made. Plasma containing various amounts of prothrombin is then prepared by dilution with saline or prothrombin-free plasma. In one experiment the curves shown in Fig. 1 were obtained.

(b) *Calcium*—Jaques and Dunlop (1945b) have shown that the use of an optimum concentration of calcium is important. As with brain thromboplastin the optimum concentration is much more limited with low levels of prothrombin. It was found to be 0.1 ml. of a 0.025 M solution as used in Quick's test.

7. The expression of results.—If the clotting time of plasma is related to its prothrombin concentration the clotting time of an abnormal plasma must be compared to that of a normal before any inference can be drawn as to its relative prothrombin content. Many authors have made this comparison by dividing the clotting time of the normal plasma by that of the abnormal and expressing the result as a percentage. The " prothrombin

index " thus obtained is based on the assumption that the concentration of prothrombin bears a linear relation to the clotting time, whereas in fact the two variables are related by a hyperbolic curve. The percentage of prothrombin must therefore be read from a calibration curve made by testing

FIG. 2.—Graphs showing the alteration in the shape of the prothrombin dilution curve with different diluents using brain thromboplastin. Prothrombin-free plasma 1 was prepared from blood collected in a silicone coated container. The plasma was obtained after centrifuging for 30 minutes at 20,000 r.p.m., and the prothrombin was removed by adsorption with an excess of freshly precipitated barium sulphate. Prothrombin-free plasma 2 was prepared from blood collected in an ordinary glass container. The plasma was obtained after centrifuging for 5 minutes at 1,500 r.p.m., and the prothrombin was removed by adsorption with the minimum amount o freshly precipitated barium sulphate.

FIG. 1.—Graphs to show alteration in clotting time observed when Quick's test is used with different concentrations of brain thromboplastin and plasma containing varying amounts of prothrombin. The prothrombin concentration was reduced by dilution with prothrombin-free plasma obtained by Seitz filtration.

samples of normal plasma in which the pro-thrombin concentration has been reduced artifici-ally. The determination of prothrombin by this technique would be relatively simple if a single reference curve could be drawn and the same curve were used by all workers. Unfortunately this is not possible because it is customary to reduce the prothrombin content by simple dilu-tion. This procedure certainly effects a quantita-tive reduction in prothrombin but unfortunately also reduces substances other than prothrombin which may be active in prothrombin conversion. Thus, if saline is used platelets and fibrinogen are lowered, and if prothrombin-free plasma is used platelets and possibly accessory factors concerned in prothrombin conversion are reduced. As Conley and Morse (1948) have shown, the refer-ence curves vary in shape according to the diluent used, and one clotting time may be taken to repre-sent a number of different concentrations of pro-thrombin according to which curve is selected as a standard (Fig. 2).

A further complication is introduced by the fact that different batches of brain thromboplastin give different coagulation times with the same normal undiluted plasma ; thus using any one diluent it is necessary to prepare a number of curves at different levels of thromboplastin activity.

Since all of the diluents produce artificial mixtures not comparable to naturally occurring prothrombin-deficient plasmas, a selection between them is arbitrary. In prac-tice we have chosen to use saline because it gives readily reproducible results in normal plasma and because of the simplicity of the processes in-volved. For standard refer-ence curves a series of saline dilution curves were made from normal plasma using different batches of brain thromboplastin which gave coagulation times from 14 to 25 seconds with undiluted plasma (Fig. 3).

It was found convenient to use a simple mathematical conversion to straight lines in place of the curves because with these it is easy to inter-polate for intermediate values. This conversion is achieved by plotting the clotting time against the reciprocal
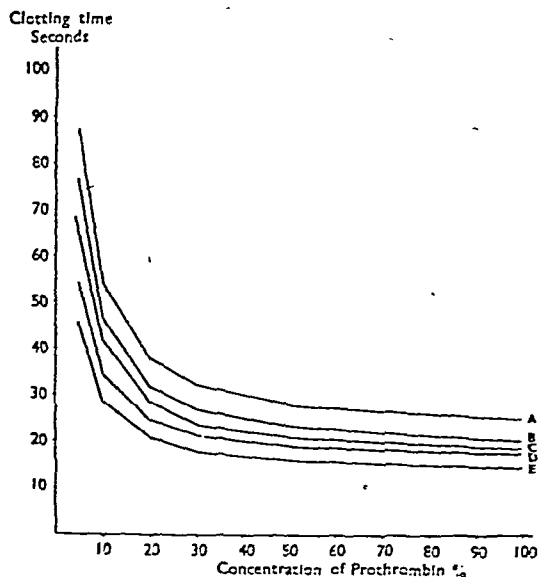
FIG. 3.—Prothrombin dilution curves obtained by testing the prothrombin time of saline dilutions of normal plasma using different batches of brain thrombo-plastin of varying potency.

of the concentration of prothrombin (Fig. 4).

Example.—Coagulation time of normal plasma: 18 seconds. Coagulation time of abnormal plasma: 42 seconds. Curve C in Fig. 4 is selected because the undiluted plasma (100 per cent) gives coagulation time of 18 seconds. Drawing a line parallel to the
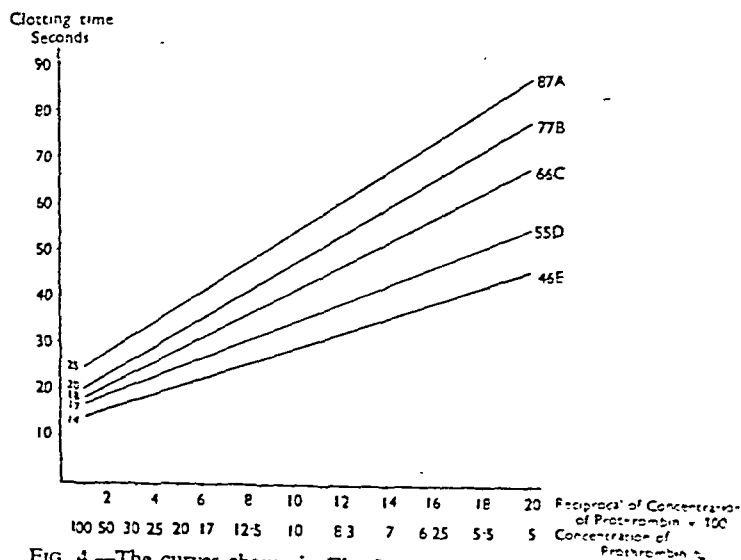
FIG. 4.—The curves shown in Fig. 3 are converted to straight lines by plotting the clotting time against the reciprocal of the concentration of prothrombin.

D

ordinate through 42 seconds it will be seen that this crosses the selected curve at a level of prothrombin equivalent to a dilution of 10 per cent in the normal plasma ; the plasma would therefore be said to contain 10 per cent of prothrombin.

This method of standardization is clearly quite arbitrary, and while the results with any one set of calibration curves will be comparable, the results from different hospitals can be related only if the reference curves are given. In much of the published work this has not been done.

(b) WITTS AND HOBSON'S TECHNIQUE

The technique is essentially similar to that of Quick except that brain thromboplastin is replaced by a 1 in 10,000 solution of Russell's viper venom (Burroughs Wellcome's " Stypven ") to 1 ml., of which 0.1 ml. of a 1 per cent alcoholic solution of crude lecithin is added. The saline dilution curves in normal plasmas are remarkably constant, and in most cases one curve will suffice for the expression of results as percentage of prothrombin. However, as with brain thromboplastin, different diluents give different curves (Fig. 5).

Case Reports

A. CASES IN WHICH THE PERCENTAGE OF PROTHROMBIN WAS ESTIMATED BY WITTS AND HOBSON'S TECHNIQUE

Case 1.—Mr. W. W., aged 53, was treated with dicoumarin for thrombophlebitis of the leg. 1,100 mg. of dicoumarin were given in the first eight days and no significant fall in prothrombin was recorded until

FIG. 6.—Diagram to show the alteration in concentration of prothrombin, as estimated with Russell's viper venom, in relation to the dosage of dicoumarin in Case 1.

the ninth day, when there was a fall to 25 per cent and a low level was maintained for four days (Fig. 6).

Case 2.—Mrs. C. D., aged 44, was treated with dicoumarin for thrombophlebitis of the leg with pulmonary embolus following a hysterectomy for fibroid. 1,100 mg. of dicoumarin were given in the first eleven days and a significant fall of prothrombin was delayed until the tenth day, when readings of 14, 30, and 15 per cent were recorded on three consecutive days (Fig. 7).

Case 3.—Mrs. E. C., aged 53, was given dicoumarin following operation for prolapse to prevent the occurrence of femoral thrombosis. She was given 700 mg. dicoumarin in the first four days, and on the fifth day had severe haemorrhage from the operation site which necessitated transfusion of 2 pints of blood. The surgeons could find no local cause for this bleeding. Her prothrombin time remained within normal limits throughout.

These cases followed a course similar to that described by Wright and Prandoni (1942), who used Russell's viper venom for prothrombin estimation. Large doses of dicoumarin were necessary to prolong the prothrombin time, the fall of prothrombin was delayed, and unpredictable haemorrhage occurred.

FIG. 5.—Graphs to show the alteration in the shape of the prothrombin dilution curves with different diluents using Russell's viper venom and lecithin as thromboplastin. Prothrombin-free plasmas 1 and 2 are the same as those of Fig. 2.

Concentration of
Prothrombin %
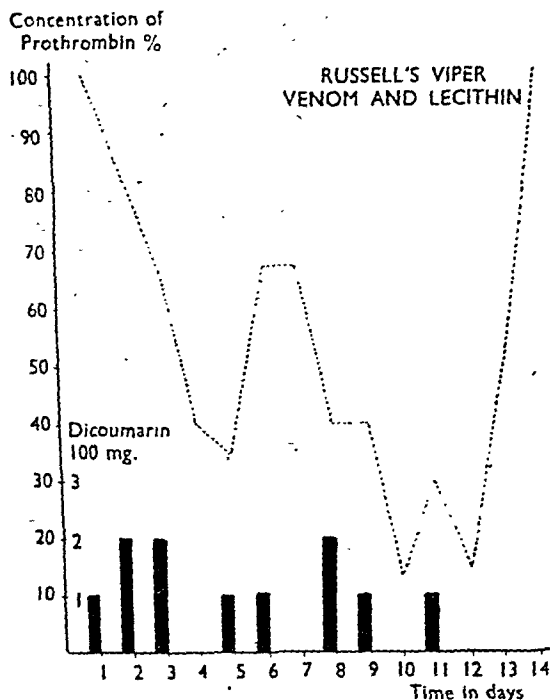
FIG. 7.—Diagram to show the alteration in concentration of prothrombin, as estimated with Russell's viper venom, in relation to the dosage of dicoumarin in Case 2.

B. CASES IN WHICH THE PROTHROMBIN WAS ESTIMATED BY BOTH WITTS AND HOBSON'S AND QUICK'S TECHNIQUES

Case 4.—Mr. E. W., aged 59, suffered from cardiac asthma and was treated with dicoumarin for thrombophlebitis of the leg. This patient was admitted to hospital twice for recurrence of this condition. On the first admission his prothrombin time was estimated by Witts and Hobson's technique. In nineteen days he was given 2,000 mg. of dicoumarin, and on one occasion only was there any appreciable lengthening of the prothrombin time. On the second admission three months later he was given 600 mg. of dicoumarin in the first three days and showed lengthening of prothrombin time by Quick's technique on the third day. The clotting time was considerably prolonged for four days and gradually returned to normal in the course of the next four days.

Case 5.—Mrs. H. F., aged 39, was treated with dicoumarin for puerperal thrombophlebitis of the leg. In this patient 900 mg. dicoumarin were given in twelve days, and on some days Witts and Hobson's technique was used and on others Quick's technique. There was a marked lack of correspondence between the results of the two methods (Fig. 8).

These cases suggest that Witts and Hobson's technique is far less sensitive than that of Quick in recording the hypoprothrombinaemia of dicoumarin therapy. In order to substantiate this probability the course of the prothrombin was followed by both techniques simultaneously in the next three cases.

Case 6.—Mr. H., aged 71, was treated with dicoumarin for thrombophlebitis following a cystotomy for benign prostatic hypertrophy. He was given 500 mg. of dicoumarin in the first two days. By Quick's technique the prothrombin dropped suddenly to 9 per cent on the third day and fell to 5 per cent on the fifth day, when there was a slight urinary haemorrhage. He was given vitamin K, the haemorrhage ceased rapidly, and the prothrombin returned to normal in four days. By Witts and Hobson's technique the prothrombin was significantly abnormal on two days only, and at the time of haemorrhage a level of 35 per cent was recorded (Fig. 9).

Case 7.—Mrs. F., aged 73, was treated with dicoumarin for thrombophlebitis of the leg following operation of the left obturator nerve. She was given 800 mg. during the first thirteen days, and the relative sensitivity of Witts and Hobson's and Quick's techniques is shown in Fig. 10.

Concentration of
Prothrombin %
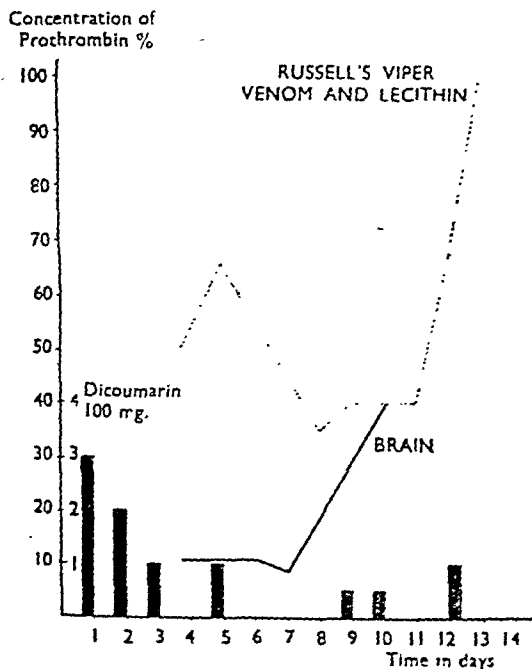
FIG. 8.—Diagram to show the alteration in concentration of prothrombin, as estimated with brain thromboplastin and Russell's viper venom and lecithin, in relation to the dosage of dicoumarin in Case 5.

Case 8.—Mr. D., aged 45, was given dicoumarin for treatment of pulmonary thrombosis following appendicectomy. 500 mg. were given in the first three days, and the patient showed an unusually marked response to the drug when the prothrombin was estimated with a brain thromboplastin (Fig. 11). With Russell's viper venom and lecithin the response was delayed and was less in extent.

The Cause of the Discrepancy between the Prothrombin Percentages Recorded with Russell's Viper Venom and Lecithin and Brain Thromboplastin

Using a two-stage technique the percentage of prothrombin estimated by the two types of thromboplastin is approximately the same (Table I). The discrepancy cannot therefore be accounted for by differences in the amount of thrombin formed. The clotting times measured by the one-stage technique are converted to so-called " percentage of prothrombin " by means of artificial dilution curves. Since the clotting times recorded by the same pathological plasma give different
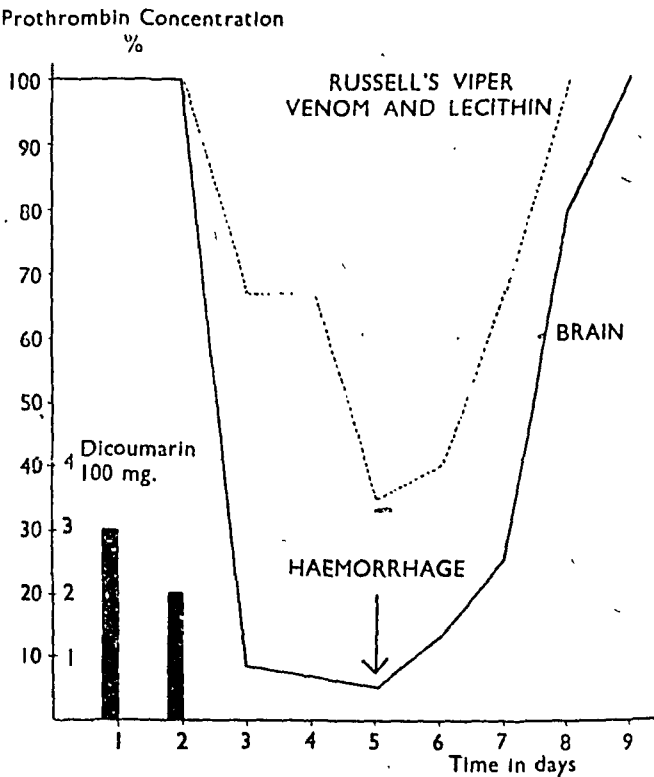
FIG. 9.—Diagram to show the alteration in concentration of prothrombin, as estimated with brain thromboplastin and Russell's viper venom and lecithin, in relation to the dosage of dicoumarin in Case 6.

FIG. 10.—Diagram to show the alteration in concentration of prothrombin, as estimated with brain thromboplastin and Russell's viper venom and lecithin, in relation to the dosage of dicoumarin in Case 7.

TABLE I

COMPARISON OF THE PERCENTAGES OF PROTHROMBIN ESTIMATED BY THE ONE- AND TWO-STAGE TECHNIQUES USING BRAIN AND RUSSELL'S VIPER VENOM AND LECITHIN THROMBOPLASTINS IN TWO PATIENTS UNDER TREATMENT WITH DICOUMARIN

| | Percentage of prothrombin | | | |
| | One-stage technique | | Two-stage technique | |
	Brain	Russell's viper venom and lecithin	Brain	Russell's viper venom and lecithin
Case 1	5	35	28	35
Case 2	22	100	55	83
	7	35	44	38
	11	100	44	41
	5	100	28	33
	9	40	25	23
	12.5	100	32	35
	26	100	27	30

percentages of prothrombin with the two types of thromboplastin, it is difficult to avoid the conclusion that the dilution curves are at fault. It has already been noted that all the diluents used reduce factors other than prothrombin, and it seems possible that one or more of these factors may have a greater effect on the speed of prothrombin conversion with Russell's viper venom and lecithin than with brain thromboplastin.
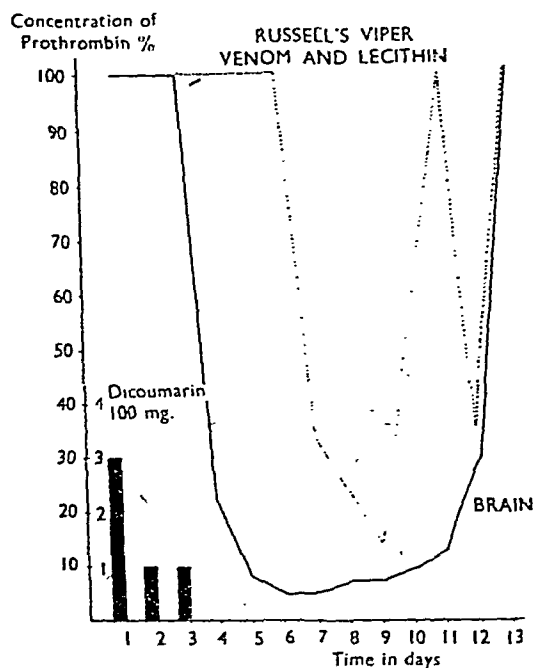
FIG. 11.—Diagram to show the alteration in concentration of prothrombin, as estimated with brain thromboplastin and Russell's viper venom and lecithin, in relation·to the dosage of dicoumarin in Case 8.

This hypothesis can be tested if normal plasma is diluted with naturally occurring prothrombin-deficient plasma in which it is assumed that only prothrombin is reduced. Curves constructed from the coagulation times of various mixtures of normal plasma with prothrombin-deficient plasmas are shown in Figs. 12 and 13. Fig. 12 was derived from a vitamin-K deficient plasma which showed only a trace of prothrombin, and in Fig. 13 a dicoumarin plasma was used which was found to contain 18 per cent of prothrombin by Quick's technique.

It will be seen that when Russell's viper venom and lecithin is used mixtures of normal plasma with naturally occurring prothrombin-deficient
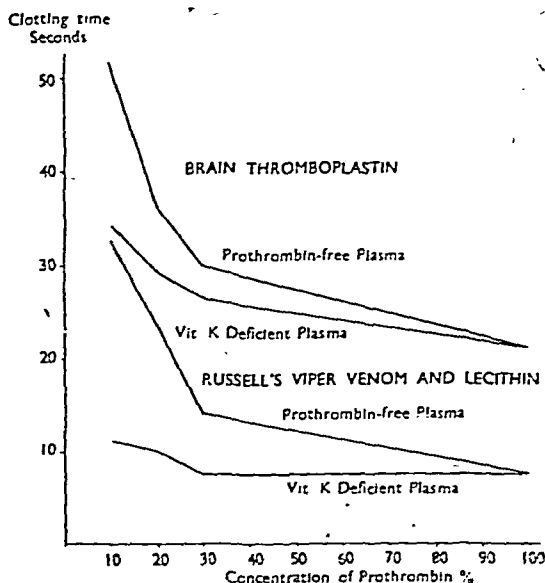
FIG. 12.—Curves to show the difference between the prothrombin dilution curves obtained by the dilution of normal with (a) vitamin-K deficient plasma, (b) prothrombin-free plasma, using brain and Russell's viper venom and lecithin as thromboplastins. The prothrombin-free plasma was made by adsorption with barium sulphate.
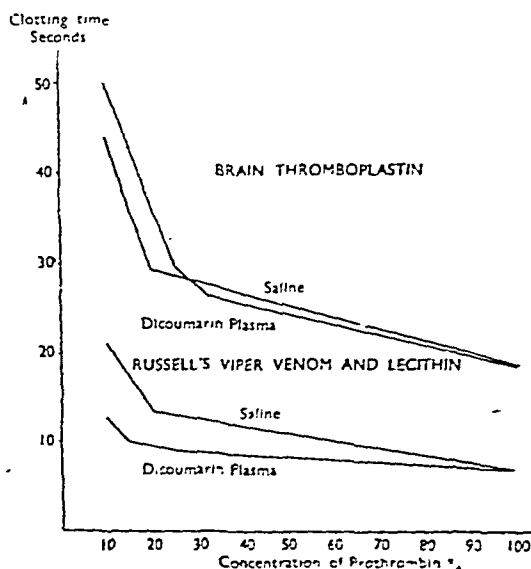
FIG. 13.—Curves to show the difference between the prothrombin dilution curves obtained by dilution of normal plasma with (a) dicoumarin plasma, and (b) saline, using brain and Russell's viper venom as thromboplastins.
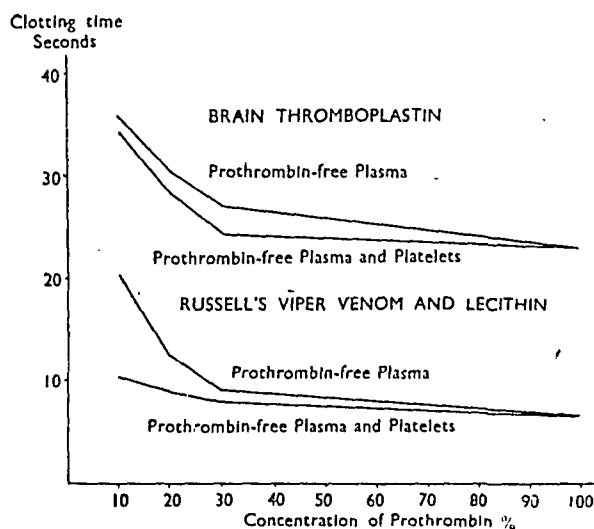
FIG. 14.—Curves to show the difference between the prothrombin dilution of normal plasma with (a) prothrombin-free plasma, (b) prothrombin-free plasma with the addition of platelets to correspond with original concentration in the plasma, using brain and Russell's viper venom and lecithin as thromboplastins. The prothrombin-free plasma was obtained by adsorption with barium sulphate.

In fact, this thromboplastin appears to measure a reduction in platelets as much as the fall in prothrombin concentration.

The extraordinary lack of sensitivity of Russell's viper venom and lecithin thromboplastin to changes in prothrombin concentration probably lies in the mechanism of thrombin generation. From the reaction of normal plasma to thrombin it can be calculated that only 5 per cent of the available thrombin that could be formed by a normal plasma is necessary for its coagulation, and it is the speed with which this level of thrombin is reached that controls the one-stage coagulation time. In Fig. 15 the early stages of thrombin generation have been followed in a normal plasma and in the plasma of a patient under treatment with dicoumarin. The minimum level of thrombin for rapid coagulation of the plasma has been indicated. With brain thromboplastin thrombin formation follows an autocatalytic type of curve, and alteration in prothrombin concentration greatly increases the time necessary for the generation of a minimum amount of thrombin. With Russell's viper venom and lecithin, on the other

plasma give much shorter coagulation times than are recorded by corresponding mixtures with saline or artificial prothrombin-free plasma. Moreover, the alteration in clotting time between 100 and 10 per cent becomes immeasurably small, giving a total range of clotting times from 7 to 11 seconds. With brain thromboplastin the discrepancy is much less marked.

The next problem that arises is which of the plasma constituents other than prothrombin is responsible for the discrepancy. One factor which is easily tested is the presence of platelets. It will be seen from Fig. 14 that when Russell's viper venom and lecithin thromboplastin is used the addition of platelets to mixtures of normal and prothrombin-free plasma causes a reduction in the coagulation time which is particularly marked at low levels of prothrombin.

FIG. 15.—Curves to show the early stage of thrombin generation in normal and dicoumarin plasma using a two-stage technique with (a) brain thromboplastin, and (b) Russell's viper venom and lecithin. The probable minimum level of thrombin for coagulation of the plasma in less than 10 seconds is indicated. The thrombin units are calculated to correspond with 1 ml. of the original plasma.

hand, an immediate liberation of thrombin occurs and the minimum level of thrombin is attained rapidly even at low prothrombin concentrations. Reduction in prothrombin content therefore makes little difference to the one-stage prothrombin time. Moreover, with Russell's viper venom and lecithin the generation of thrombin is so rapid that the sensitivity of the one-stage technique is almost certainly limited by the speed of the thrombin fibrinogen reaction.

If the discrepancy between Russell's viper venom and lecithin and brain thromboplastin results from a differential sensitivity to factors other than prothrombin, a similar difference should occur in all types of hypoprothrombinaemia. In a limited series of pathological cases such a discrepancy was found (Table II).

TABLE II

PERCENTAGE OF PROTHROMBIN ESTIMATED WITH BRAIN THROMBOPLASTIN AND RUSSELL'S VIPER VENOM AND LECITHIN IN VARIOUS PATHOLOGICAL CONDITIONS

Condition	Prothrombin concentration	
	Using brain thromboplastin (%)	Using Russell's viper venom and lecithin (%)
Cirrhosis of the liver	35	30
"	14	50
"	35	80
"	50	100
"	11	35
"	20	80
Vitamin K deficiency	25	100
"	Clotting time 20 min.	Clotting time 56 sec.
Ulcerative colitis ..	25	35
Treatment with quinidine sulphate	30	50

Discussion

Much of the confusion aroused by the estimation of prothrombin follows from an unwarranted belief that the one-stage test can give a real measure of prothrombin concentration provided that sufficient care is taken in standardization of the technique. From the experiments described here it is clear that this is not possible. Not only do the two thromboplastins, each carefully standardized, give different results, but each thromboplastin can be made to give a series of differing results according to which of the diluents is used to prepare the calibration curves. The technique cannot therefore measure prothrombin, but gives

a relative measure of the speed of production of the small amount of thrombin necessary for coagulation. It might save confusion if all pretence at measuring " prothrombin percentage " were abandoned and the results were expressed as " relative prothrombin efficiency " or, as was suggested by Witts (1942) and Witts and Hobson (1942), an " accelerated clotting time " in percentage of normal.

The value of the test depends essentially on its ability to predict a haemorrhagic level. Bollman and Preston (1942) have shown that haemorrhage following dicoumarin administration is not directly proportional to the "concentration of prothrombin." In the patients described here it is clear that Cases 3 and 6 had a particular predisposition to the haemorrhage. Fortunately haemorrhage often fails to occur with a very low content of prothrombin, and Barker and others (1945) have claimed that haemorrhage was uncommon with prothrombin levels above 10 per cent by Quick's technique. In Case 6 the " prothrombin concentration " was 5 per cent at the time of bleeding, and haemorrhage rapidly ceased when it was raised above 10 per cent. With Quick's technique, therefore, it is probable that if the " concentration of prothrombin " is maintained above 10 per cent no uncontrollable haemorrhage will occur. The value of this test rests on the vast clinical material in which it has been used rather than on the soundness of its theoretical foundations.

With Russell's viper venom and lecithin, on the other hand, no certain haemorrhagic level can be defined because a sufficient number of cases have not been recorded for any such conclusion to be reached. Moreover, the technique is dangerously insensitive to alterations in prothrombin concentration, and in a routine laboratory it is impossible to measure the one or two seconds differences in coagulation time that may bring the patient within the haemorrhagic level.

It is surprising that in an annotation in the *British Medical Journal* (1947) doses of 100 to 300 mg. of dicoumarin daily are recommended, and in a further annotation (1948) mention is made of the daily administration of 200 mg. These amounts are dangerously high and would certainly cause haemorrhage in patients such as Cases 3 and 6 with a predisposition to bleeding.

Summary

1. Russell's viper venom with lecithin is frequently used to replace brain thromboplastin for the estimation of prothrombin in the one-stage technique.

2. The assumption that Russell's viper venom and lecithin · will give the same results as brain thromboplastin is not justified, by theoretical considerations or by experience of its use in practice.

3. One pathological plasma will record a different percentage of prothrombin when tested with brain and Russell's viper venom thromboplastins even when the results are read from appropriate calibration curves.

4. The calibration curves are drawn from artificial plasma mixtures which are not comparable to naturally occurring prothrombin-deficient plasmas. The two thromboplastins have a different sensitivity to the reduction of factors other than prothrombin which are lowered in the artificial mixtures.

5. The one-stage technique using brain thromboplastin is of proved value but cannot measure prothrombin concentration, and its results would be better recorded as " relative prothrombin efficiency " or as an " accelerated clotting time " expressed as a percentage of normal.

6. Russell's viper venom and lecithin cannot be used to replace brain thromboplastin as a routine procedure because it is not sufficiently sensitive to alterations in prothrombin concentration.

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PROTHROMBIN TIME IN DICOUMAROL THERAPY

BY

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In the course of controlling dicoumarol therapy given (*a*) as a postoperative routine to prevent thrombosis (fifty-five cases) and (*b*) therapeutically in cases of established venous thrombosis (nine cases), certain peculiarities have been observed in the behaviour of the prothrombin time, particularly in relation to dicoumarol haemorrhage. Two sources of thromboplastin have been used, Russell viper venom and human brain. Data are presented which suggest that the brain extract is to be preferred to venom, for estimations of prothrombin levels are then more in accord with clinical observation.

Technical Methods

With Russell viper venom.—This method is essentially that of Fullerton's (1940) modification of Quick's one-stage method, with the slight additional modifications noted below:

2 ml. of venous blood was placed in a tube containing 1.6 mg. of potassium oxalate and 2.4 mg. ammonium oxalate, shaken well, and centrifuged at 1,500 r.p.m. for 5 minutes, and the plasma is used in the test. Fullerton used liquid sodium oxalate, but providing that the quantity of blood put in the tube with the solid oxalate is not less than 1.5 ml. and not more than 2.5 ml. the results are the same.

The test.—0.1 ml. of oxalated plasma and 0.1 ml. of 1/10,000 " Stypven " solution were placed in a tube in a 37° C. water bath and left for 6 minutes (see below). 0.1 ml. of M/40 calcium chloride at 37° C. was added quickly, and the time taken until the appearance of fibrin particles was noted. All estimations were performed in triplicate.

The tubes containing oxalated plasma and Stypven solution were left for 6 minutes at 37° C. because it was found that up to 4 or 5 minutes at 37° C. the prothrombin time increased fairly rapidly, and reproducible results could not be obtained, while between 5 and 9 minutes little change occurred, and consequently results were more reliable. This change is probably due to two factors: (i) destruction of Quick's labile factor, and (ii) the proteolytic activity of the Stypven (Hobson and Witts, 1941).

With human brain extract.—The method is exactly the same as that described by Aggeler and others (1946). Points to be watched are: (*a*) the brain extract

must be very finely ground, as, if the particles are too large, they settle in the saline too rapidly, leaving too clear a supernatant fluid, which is almost devoid of thrombokinase activity—it appears that this activity resides in the particles themselves; (*b*) over-incubation of the brain extract in saline before use must be avoided—12 minutes at 50° C. is ample; activity falls rapidly after 15 minutes at this temperature.

The prothrombin content of the plasma was read off on a graph of prothrombin time in seconds against percentage plasma concentration (in saline diluent).

The graphs used were prepared from the first patients in the series, twenty cases for the venom method, ten cases for Quick's. As the curves were all the same shape for the different patients, though shifted up or down the ordinate according to the individual variation, it was found possible. instead of preparing a fresh curve for each patient, to use one curve for the venom method and one for Quick's method, using parallel curves to allow for the variation.

General management of experiment.—Each case was tested before and after operation as a routine; dicoumarol was started on the fourth postoperative day (300 mg. on the first evening, 200 mg. on the next); and in the morning after the two doses, counted as " day 2," the prothrombin time was estimated, and thereafter every other day until treatment was finished and the patient was ambulant. Further tests were carried out two or more days after cessation of therapy to see how quickly the prothrombin levels returned to normal. Patients requiring emergency operation or cases of established phlebothrombosis were tested before treatment and thereafter in the same manner as above.

Results

Patients before operation.—It was observed that the prothrombin time of 55 patients before gynaecological operation varied between 10 and 21 seconds with the venom method. This variation is somewhat greater than has been found in previous investigations on normal subjects. For instance, Fullerton (1940) found a variation of 18 to 25 seconds, while Witts and Hobson (1942), using venom and lecithin, found one of 8 to 12 seconds. Aggeler and others (1946). in a full

statistical study of 30 normal subjects, using Quick's technique but with human brain extract, found a variation of only 10 to 13 seconds, whereas Nygaard (1941), also using Quick's technique but with rabbit brain extract, found a variation of 11 to 19 seconds.

Three subjects of the present series tested three or more times over a period of three to six months have remained constant to within 3 seconds of their original times, and Witts and Hobson suggested in their paper (1942) that the variations found in different individuals tested by the same method represented a true variation in the coagulability of normal plasmas.

Patients under dicoumarol therapy

USING RUSSELL VIPER VENOM.—Under dicoumarol therapy the prothrombin levels fell more or less as expected, but it was soon found that patients were bleeding at levels higher than those usually regarded as dangerous. Quick has stated that the prothrombin level falls to below 10 per cent before haemorrhage occurs, whereas in the first series of thirty cases controlled only by the venom method seven patients bled when their prothrombin levels were much higher than this—24 to 45 per cent.

Two patients developed severe vaginal haemorrhages and required transfusion. However, both had amputations of the cervix, after which it is almost impossible to obtain adequate covering of the raw area. On the other hand, several patients subjected to this operation have been treated without resulting haemorrhage. Four patients bled from sites not involved in the operation.

It was noted that in half the cases the prothrombin levels continued to fall for 2 to 3 days even though the haemorrhage had ceased.

USING QUICK'S METHOD PARALLEL WITH THE VENOM METHOD.—In an endeavour to find a more reliable method, Quick's technique was tried in conjunction with the venom method on the blood of the next patient who bled, and, as pointed out by Lempert (1948), the prothrombin level was found to be much lower as estimated by Quick's method than by the venom method.

In view of this, it was decided to do the two tests in parallel on the next twenty cases. This has been done, and the results in ten patients are summarized in Fig. 1. The ten cases not recorded on this graph had had longer courses of dicoumarol or had tests at less regular intervals, but they all showed similar features.

The behaviour of the prothrombin level.—In every case, on and after the second day of dicoumarol administration the prothrombin level fell much more rapidly as estimated by Quick's
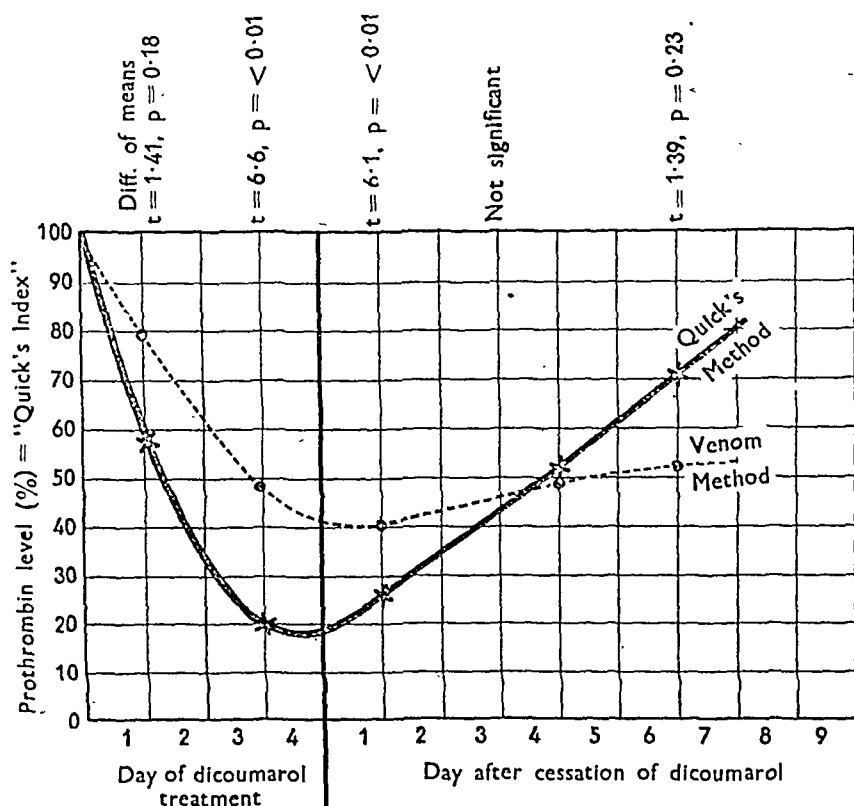
FIG. 1.—Graph showing response to dicoumarol of the prothrombin level as estimated by Fullerton's and by Quick's methods. Made from figures obtained from ten patients who had sufficient estimations and courses of dicoumarol of similar duration. All other cases showed exactly similar responses, but owing to clinical exigency such as variations in duration of treatment, etc., they could not be included in the construction of the graph. The differences between the readings on the graph are statistically significant at the point of inflexion of the curves, that is, after two days on dicoumarol and for three days after stopping the drug, when the curves approach each other.

method than by the venom method, and in every case the prothrombin level returned towards normal far more rapidly (with due allowance for the lower level to which it had fallen) when estimated by Quick's method (Fig. 1).

Haemorrhages.—Three cases in this series had haemorrhages, all slight.

The prothrombin level of Case 33 had fallen by the seventh day to 8 per cent (Quick's) or 24 per cent (venom), and dicoumarol was accordingly stopped. She was well until the evening of the ninth day, when she complained of some pain in the loins, and next morning slight haematuria was noticed. This lasted only one day, and the next day her prothrombin level had risen by Quick's method to 20 per cent, while by the venom method it had shown no improvement, being 23 per cent, although the bleeding had ceased.

Case 41 had a sudden haemorrhage of 4 oz. from her abdominal wound on the ninth postoperative day, after only 700 mg. of dicoumarol. The blood appeared stale, and oozing of dark blood continued for some days. The clinician thought it was a haematoma. The patient's prothrombin level was 28 per cent by Quick's and 65 per cent by the venom method.

Case 55, who had had a vaginal hysterectomy, had slight vaginal haemorrhage on the ninth postoperative day, lasting for two days, and also some fever (99.6° F.), both of which manifestations the surgeon thought were due to a mildly infected operation site. A slight oozing from the venipuncture hole suggested an overdose of dicoumarol. Her prothrombin level was 28 per cent by Quick's and 50 per cent by the venom method.

Discussion

In view of the more rapid fall in prothrombin level when estimated by Quick's method, less dicoumarol was required to keep the prothrombin level between 10 and 30 per cent, and consequently fewer haemorrhages occurred.

It may be argued that the levels given by the venom method are the correct ones, and that if one aimed, say, at a level of 40 to 60 per cent prothrombin adequate control could be obtained. However, in view of the slower response with the venom method, adequate warning of impending haemorrhage is not given and cases cannot be controlled so accurately.

The object of this treatment is to prevent thrombosis, and it may be argued that the smaller dosage of dicoumarol required to reduce the prothrombin level to 30 per cent using Quick's method gives insufficient protection against this danger. We have had one case of venous thrombosis in the latter series: in a woman who had a vaginal hysterectomy and was very septic, with fever and discharge. It occurred after 1,300 mg. dicoumarol, when her prothrombin level was about 40 per cent (Quick) and 35 per cent (venom) two days after cessation of dicoumarol therapy and when the patient was getting up. The thrombosis was a slight one, and with two doses of heparin and more dicoumarol no further trouble ensued. The correlation between prothrombin levels by the two methods, clinical findings, and dicoumarol administration in this case is seen in Fig. 2, which illustrates well the more lively response of Quick's method to administration or withdrawal of dicoumarol, and the value of daily estimations of prothrombin time in controlling treatment.

Though the venom methods of prothrombin-time estimation may give moderately reliable figures on normal plasmas, they do not follow the true prothrombin level (or at least the blood coagulability) sufficiently closely to give warning of impending haemorrhage, and the methods using acetone dried brain extract as a thromboplastin are far more reliable in this respect. In fact there
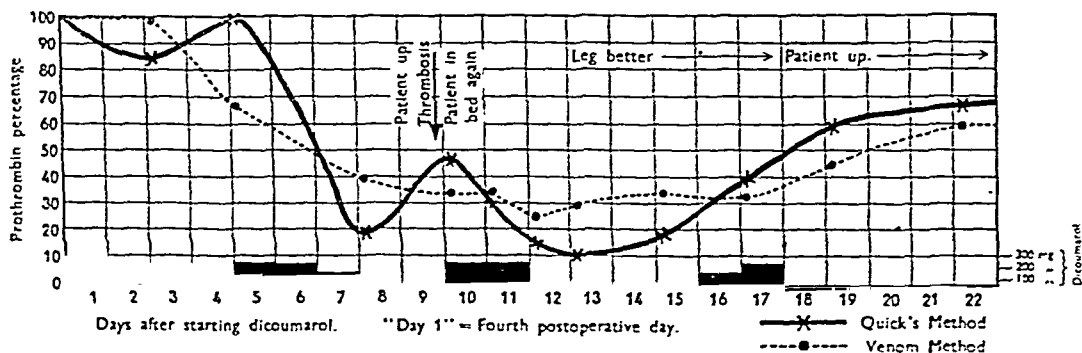
FIG. 2.—Graph of the prothrombin level of Case 53, showing time-relations of dicoumarol administration, thrombosis, etc., to changes in prothrombin level as measured by Quick's and Fullerton's methods.

seems to be a delay of two or three days in the prolongation of prothrombin time as estimated by the venom method, so that prolongation occurs after the haemorrhage has taken place ; and in some cases there is very little prolongation even though severe haemorrhage has occurred.

Neither of these serious defects has been observed with the brain extract method, so that, for the safety of the patients and the peace of mind of the pathologist, a return to Quick's method seems long overdue.

Summary

While controlling the treatment of patients on dicoumarol with Russell viper venom method of prothrombin-time estimation, it was noted that some patients bled with prothrombin concentrations anywhere between 24 and 45 per cent.

It was found that Quick's method was more sensitive to changes in prothrombin in patients on dicoumarol, and that it provided a more accurate forecast of possible haemorrhage. A return to this method is advocated.

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CELLULAR ANALYSIS OF THE ASPIRATION LUNG BIOPSY FROM NORMAL AND SOME PATHOLOGICAL CONDITIONS

BY

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In a previous report (Godlowski, 1948) special attention was drawn to the obscure role which the eosinophils played in allergic conditions, in particular in allergic bronchial asthma treated with insulin hypoglycaemia and adrenaline infusion during which a peripheral eosinopenia occurred and in both of which the mechanism of the eosinopenia seemed to be identical. There is on record (Bertelli and others, 1910) certain evidence that in dogs big doses of adrenaline produce a peripheral eosinopenia with local eosinophilia in the liver. Analysis of the bone-marrow in allergic conditions shows no substantial alteration in the eosinophil content (Godlowski, 1948). In order to try to trace the fate of the eosinophilic cells during the adrenaline infusion and insulin hypoglycaemia it was decided to do a lung aspiration biopsy, assuming the lung to be the presumptive shock organ and the place where eosinophils assemble during the peripheral eosinopenia.

Aspiration lung biopsy has been done by many clinicians (Stewart, 1930; Sappington and Favorite, 1936) for the bacteriological identification of the bacteria causing lung infection, particularly of the pneumococcus in pneumonia for ascertaining the proper serum treatment. In spite of favourable reports given by these and other clinicians who have used this method and who have stressed its harmlessness and its great diagnostic value, this method is at present not in general use in lung diagnosis except in cases of lung tumours (Martin and Ellis, 1930; Stewart, 1930; Sappington and Favorite, 1936; Wilson, 1945). Serious objections to this method are advanced only by those who have had inadequate statistical data. Their objections are based mainly on somewhat theoretical

arguments and fear of spreading infection along the puncture canal, of injury of the large pulmonary vessels, and of production of pneumothorax, air embolism, etc. It has been shown, however (Stewart, 1930; Martin and Ellis, 1930; Bullowa, 1936), that these complications occur so rarely that they need not be regarded as discrediting this method of diagnosis in lung diseases. Two thousand cases of lobar pneumonia, for example, were complicated by empyema in 4.6 per cent of cases subjected to lung biopsy, whereas 1,913 cases of lobar pneumonia without lung aspiration biopsy showed empyema in 5.1 per cent—that is, the frequency of empyema was of the same degree in both series. Thus the empyema must be considered as a complication not of the lung aspiration biopsy but of the lobar pneumonia (Sappington and Favorite, 1936).

Method

Seventy-five clinically normal men and women, of whom fifty were tobacco smokers and twenty-five non-smokers, with negative x-ray reports of the chest, and ten cases with various pathological conditions of the lung, were subjected to lung aspiration biopsy. The first twelve were premedicated with 0.02 g. of morphia and 0.001 g. of atropine sulphate, injected subcutaneously thirty minutes before biopsy. Since a certain number of cases, however, responded with toxic symptoms which upset them more than the biopsy itself, the premedication was thereafter dropped and attention concentrated on a local anaesthesia, to which very great importance is attached. The local anaesthesia consisted of 2 per cent solution of procaine hydrochloride. An intradermal wheal was made in the first instance and then the tissues of the chest were gradually infiltrated as deep as the costal and

finally visceral pleura and the adjacent parts of the lung tissue itself. Very great importance is attached to the anaesthesia of the pleura, since the literature includes cases in which simple tapping of the pleura caused so-called vasovagal syncope, leading in some cases to instantaneous death (Stewart, 1930 ; Sappington and Favorite, 1936). Local anaesthesia of the pleura can substantially minimize the danger of the vasovagal syncope. Five to ten minutes after the injection of procaine hydrochloride the patient's chest was again screened by way of a repeat control. The site of the biopsy is of no importance in normal cases ; in pathological cases the aspiration biopsy should be done at the most easily accessible place of the radiologically discovered pathological alteration ; in the present series of biopsies it was made in the sixth or seventh intercostal space in the anterior axillary line of the right lung. Two per cent tincture of iodine was used for disinfection of the skin.

In most of the cases presented here an ordinary large lumbar puncture needle was used for the lung biopsy. As, however, among the biopsy elements there were found cells suspected to be of skin or pleural origin, the two-needle method was applied in order to avoid the inclusion of such cells and to obtain a film of pure lung elements. One short needle of large calibre with the stilette in it was pushed through the whole chest wall and both pleurae, and, as soon as the needle appeared in the lung itself (the whole procedure being controlled by radiograph), the stilette was removed and a second needle much longer and thinner than the first and also with a stilette in it was passed through the first needle. As soon as the second needle appeared in the lung the stilette was removed and a 20 ml. syringe was connected to it. By making an intense, sharp aspiration the second needle was pushed into the lung at times as deep as 20 cm. and moved in and out to the tip of the first needle several times. During this procedure the patient must stop breathing in order to avoid laceration of the lung by respiratory movements with subsequent pneumothorax. The aspiration was stopped at the point where the second needle on the way out approached the tip of the first one ; the second needle was then slowly drawn through the first one. The content of the second needle was spread on the glass slide and, if the amount was sufficient, a film was made of it. The first needle was removed and the place of the puncture dressed. In spite of these precautions the lung specimen so obtained in a few cases still contained pleural cells, which means that the second needle was contaminated by the material adherent to the wall of the first needle. To avoid any incidental contamination from the skin, an incision was, in a few cases, made in the skin and the two-needle method used through the wound. After a week a routine x-ray control of the chest was always made.

A biopsy smear obtained in this way was dried for a period of one to twelve hours at room temperature and was stained by the Leishman or Jenner-Giemsa method. Iron haematoxylin or mucicarmine staining gives very poor differential value and for this reason is not recommended.

The precaution of using the two-needle method in lung biopsy was necessary only for identification of certain cells and exclusion of cellular elements of the pleura and skin. For ordinary diagnostic purposes the one-needle method is entirely satisfactory if it is kept in mind that the cells described below belong to the serosa of the pleura. The detailed description of the one-needle method may be found elsewhere (Martin and Ellis, 1930, 1934).

If the local anaesthesia is well performed the majority of the patients feel only negligible pain during the whole procedure. More sensitive individuals, however, sometimes feel a short stabbing pain which in one or two cases may persist for a few hours ; such pain is not intensive and can easily be allayed by light analgesics. Out of eighty-five normal and pathological men and women in the present series, one had a slight haemoptysis which ceased after twelve hours. Pneumothorax occurred in three : it was small

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Fig. 1.—Normal pulmogram of a non-smoker showing few macrophages (" dust cells "), large cells with pale-blue cytoplasm, and alveolar histiocytes with dark-blue cytoplasm ; in both types of cells are seen few particles in their cytoplasm. The other cells seen in the film are of the type seen in the peripheral blood. (Leishman, ×100.)

Fig. 2.—Two macrophages (" dust cells ") from Fig. 1. (×800.)

Fig. 3.—Alveolar histiocytes from Fig. 1. (×800.)

Fig. 4.—Sheet of nucleated alveolar epithelium from a normal pulmogram ; their cytoplasm free from any particles, there are some vacuoles seen in the cytoplasm. (Leishman, ×800.)

Fig. 5.—Two macrophages loaded with haemosiderin (" heart-failure cells ") from a case with chronic venous congestion in the lung (mitral stenosis). (Jenner-Giemsa, ×800.)

Fig. 6.—Two mesothelial cells from the pleura from a normal pulmogram. (Leishman, ×800.)

Fig. 7.—Pulmogram of a heavy tobacco-smoker showing very numerous macrophages with pale-blue cytoplasm (" tobacco cells ") and alveolar histiocytes with dark-blue cytoplasm, both heavily packed with particles. There is also one giant cell. (Leishman, ×100.)

Fig. 8.—One giant cell surrounded with " tobacco cells " and alveolar histiocytes from the case seen in Fig. 7. (Leishman, ×800.)

Fig. 9.—Tobacco cells and alveolar histiocytes packed with particles from the film seen in Fig. 7. (Leishman, ×800.)

Fig. 10.—Ciliated epithelium with three goblet elements forming a palisade layer. Also two " heart-failure " cells. From a case of chronic venous congestion of the lung. (Jenner-Giemsa, ×800.)

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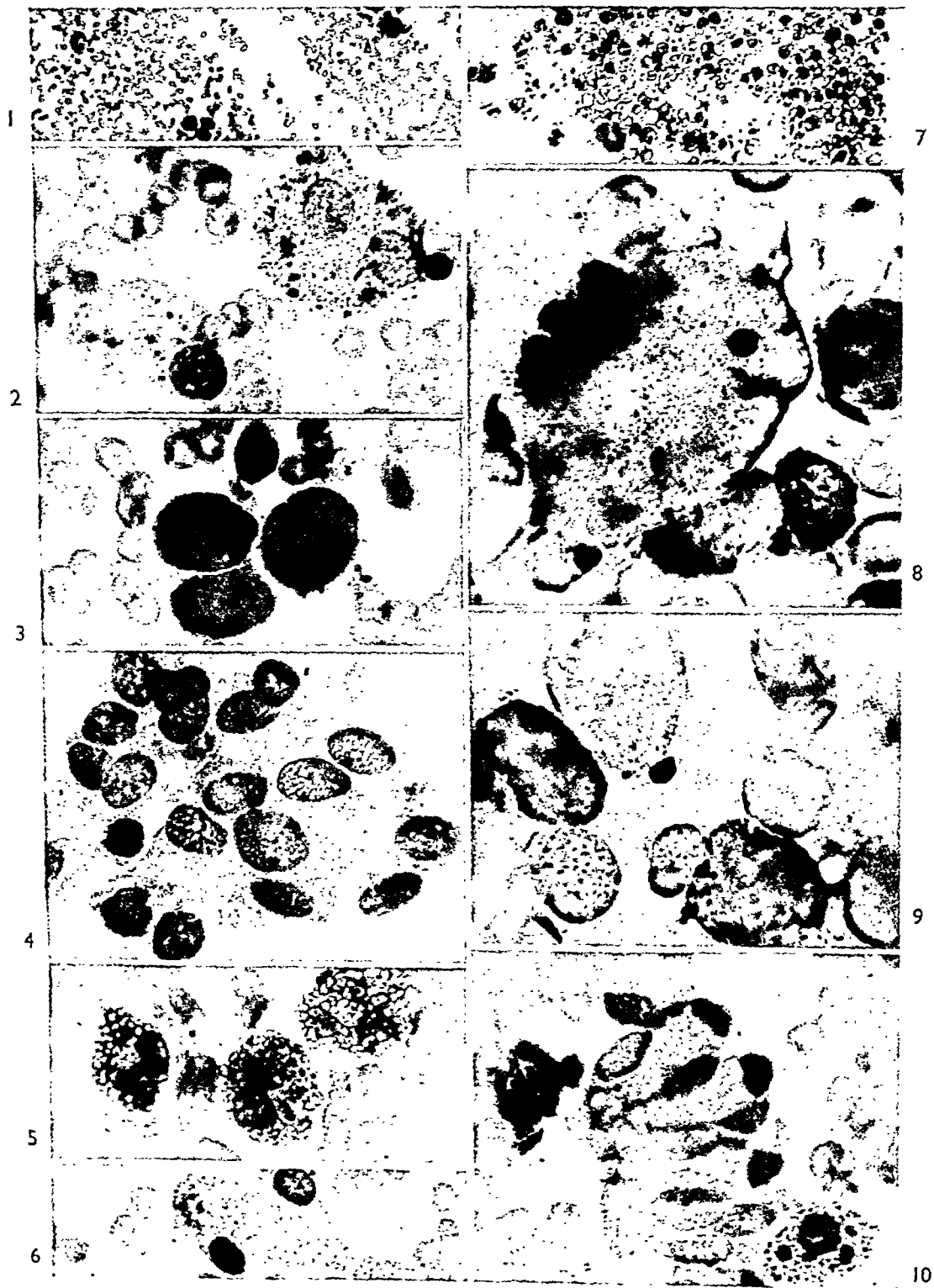
and was discovered only by routine x-ray control; the partly collapsed lung re-expanded totally in fourteen days.

Results and Discussion

A pulmogram usually consists of cells derived from (1) lung tissue, (2) blood aspirated from the pulmonary vessels, and (3) tissues of the thoracic wall and pleura.

The identification of some of the pulmonary elements as regards origin is very difficult and sometimes impossible. Therefore the terminology used in this paper for the cells concerned is based only on the close resemblance of the cells in pulmogram to the elements described by histologists. Histological terminology is, however, not in the least unanimous about the nature of the alveolar lining or the origin of macrophages.

1. **Elements derived from the lung tissue itself** are: (a) macrophages, (b) histiocytes of the alveolar lining, (c) nucleated alveolar epithelial cells, (d) "non-nucleated plates," (e) reticulum cells, (f) collagenous and elastic fibres, (g) lymphoid cells, (h) ciliated and non-ciliated epithelium from the bronchi and bronchioles.

(a) *Macrophages* vary greatly in size (15 to 50 microns in their longer diameter) and in shape, being round, oval, or irregular. Their cytoplasm with Leishman or Giemsa staining is pale blue; it may be packed with particles of different origin, shape, and size, or might have only a few granules or none at all. Their nuclei are round or oval, may be single or multiple, and stain a violet colour; they contain one or more nucleoli and are abundantly filled with coarse, granular chromatin. Cells with several nuclei and reaching the upper limits of the dimensions specified above are rarely seen in normal pulmograms; the cytoplasm of these very big cells often contains particles, but by this method of staining it is difficult to decide whether they include any bacteria. These macrophages belong to the class of giant cells (Figs. 7 and 8). The function of the macrophages is phagocytosis, and according to the phagocytized material they have acquired different names; if they contain dust or carbon particles they are called "dust cells" (Figs. 1 and 2), or if they include granules of haemosiderin derived from ingested red cells phagocytosed in chronic venous congestion of the lung they are called "heart-failure cells" (Figs. 5 and 10); they may also be called "tobacco cells" (Fig. 9) if they contain partly or totally carbonized tobacco.

The origin of these cells is not conclusively elucidated; Lang (1925) and Gazayerli (1936) suggest that they arise from the "septal cells" by losing contact with their ground and growing in size. Others suggest an origin from the nucleated alveolar epithelium (Cappell, 1923, 1929; Carleton, 1927) or from septal pericapillary cells or the mononuclear cells of the blood (Foot, 1927).

The "dust cells" in a normal pulmogram are usually irregular in their distribution and are found most plentifully at the edges of the film, where they are suitable for the observation of qualitative alterations only. To get information about quantitative changes in the "dust cells" it is advisable to compare the number found in the middle fields of the smear with those on its edges, as is done in a differential count of the peripheral blood. A pulmogram may be considered normal as regards quantitative changes when the number of "dust cells" found in one high-power field (an average of 100 fields) does not exceed three to five macrophages. If, however, peripheral blood were aspirated in large quantities into the syringe, the quantitative estimation would be fallacious.

(b) *Histiocytes of the alveolar lining* (Fig. 3) are smaller cells than "dust cells" and more regular in shape, with highly basophilic cytoplasm in which are seen particles of different size and shape. The single nucleus is as a rule eccentrally located, oval or round in shape, and densely filled with granular chromatin.

Gazayerli (1936) in his experiments on animals and in human beings found cells with high phagocytic power between the nucleated alveolar epithelium. These occasionally showed mitosis and were regarded by him as capable of being shed into the lumen of alveoli. Such cells may resemble those reproduced in Fig. 3.

(c) *Nucleated alveolar epithelial cells* (Fig. 4) are the cells much smaller than "dust cells," and their shape is polygonal if they are in sheets or more round if isolated. The blue cytoplasm with small violet patches is never granular but may be finely vacuolated. The large single nucleus filled with coarse granules of chromatin is violet and usually contains one or more nucleoli.

The lack of any particles in their cytoplasm in biopsies in which phagocytes are packed with granules proves their total inability to act as phagocytes. The amount of the nucleated alveolar epithelium might in certain normal pulmograms be greater than all other elements. Cells of this nature have been identified as those which line the walls of the alveoli themselves and are thus part of the interalveolar septum (Gazayerli, 1936; Miller, 1947).

(d) "*Non-nucleated plates*" (Fig. 11) are very thin structureless plates which are polygonal or

TABLE

AVERAGE PER CENT VALUES OF DIFFERENTIAL COUNTS MADE FROM CAPILLARY BLOOD AND FROM ASPIRATION LUNG BIOPSY FROM THIRTEEN NORMAL CASES

| | Neutrophils | | | | Eosinophils | | Basophils | | Monocytes | | Lymphocytes | |
| | Young | | Mature | | | | | | | | | |
	Capillary blood	Lung	Capillary blood	Lung	Capillary blood	Lung	Capillary blood	Lung	Capillary blood	Lung	Capillary blood	Lung
Differential counts: % values ..	2.11	2.22	63.08	64.77	2.38	3.15	0.23	0.23	5.69	3.48	27.38	26.85
Standard error	±1.1	±1.31	±10.14	±7.61	±1.73	±2.03	±0.42	±0.42	±2.49	±2.57	±9.9	±9.9

quite irregular in outline and stain very lightly bluish violet; they are usually located at the thinner end of the smear singly or in groups. The two-needle method, made through the incision in the skin, on the one hand and the aspiration lung specimen taken at necropsy on the other prove their lung origin conclusively.

Similar plates have been described by Kölliker (1881) and Lang (1925) as forming part or all of the alveolar lining. In the aspiration lung biopsy, however, they may be an artifact—for example, cells of various kinds from which the nucleus has been removed by manipulation while making the film.

(e) *The reticulum cells* (Fig. 12), which are rarely found in a normal pulmogram, are branching elements with deep blue non-granular cytoplasm and hyperchromatic nucleus, situated in the middle of the body of the cells. They are quite numerous in a pulmogram from pathological conditions of the lung entailing destruction of the pulmonary tissue.

(f) *Collagenous and elastic fibres* are elements often met in pulmograms obtained from a lung involving destructive processes; both are stained violet by this method; collagenous fibres (Fig. 12, c.f.) are thick and twisted threads and the elastic fibres (Fig. 12, e.f.) are fine straight ones.

(g) *Lymphoid cells* are cells of young lymphocytic type as met in the peripheral blood, with a round violet nucleus and one nucleolus; the pale blue cytoplasm never contains any particles and in this respect resembles the nucleated alveolar epithelium. They are, however, much smaller in size than alveolar epithelium, while the nuclear chromatin is more compact and the nucleus itself also is much smaller.

(h) *Ciliated and non-ciliated epithelial cells* (Fig. 10) lining bronchi and bronchioles of different calibre are very seldom seen in a normal biopsy. Pathological conditions such as venous congestion and chronic bronchitis, however, are characterized by the appearance of such ciliated cylindrical cells arranged in palisade formation or singly. Cilia by this method of staining are pink-red or violet in colour. If arranged in a layer, these cells may include goblet elements (Fig. 10). The bottom of the row shows polygonal epithelial cells with oval nuclei and they are the parent cells of the surface epithelium.

Non-ciliated epithelial cells lining respiratory bronchioles are cells of cuboidal outline with a large oval nucleus and cytoplasm free of any granulation in normal conditions, whereas in pathological ones such as inflammations the cytoplasm may be vacuolated.

2. **White blood cells aspirated from the pulmonary vessels.**—The differential count of these cells was compared with capillary blood differential count. The results are shown in the Table and indicate that difference between the peripheral and pulmonary differential counts lies within the limits of experimental error.

3. **Cells derived from tissue of the thoracic wall.** —These are mainly those from the costal and visceral pleura. Such mesothelial cells, (Fig. 6) may be seen in the pulmogram obtained by the one-needle method. As was mentioned before, however, they may also appear in pulmograms made by the two-needle method, but in much smaller number. They are scattered as loose cells over the whole film. Their pale violet-blue cytoplasm is free of granulations. Their round, violet nucleus is centrally or eccentrally localized and has thick granular chromatin.
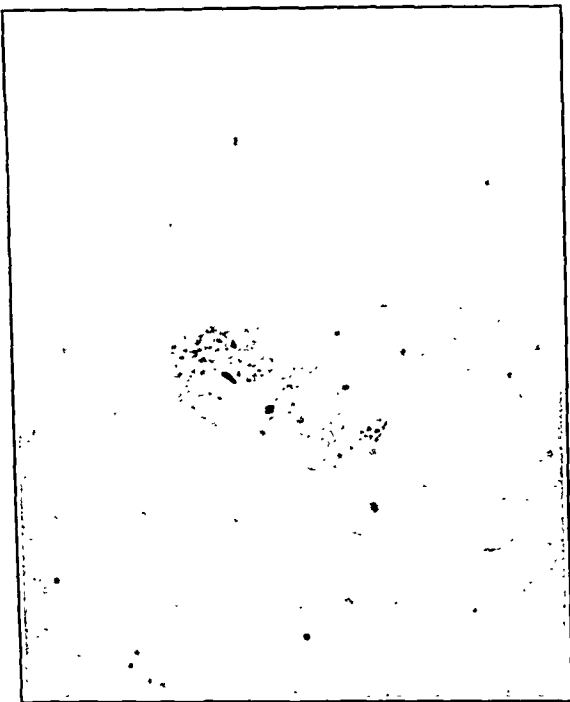
FIG. 11.—Two "non-nucleated plates" of a polygonal shape without any structure in their body (the granules seen are artefacts). (Leishman, × 800.)

FIG. 12.—Pulmogram from an ulcerative lung tuberculosis showing numerous cells of the lymphocyte and poly-morph type and some collagenous (c.f.) and elastic fibres (e.f.) and also few reticulum cells (r.c.).
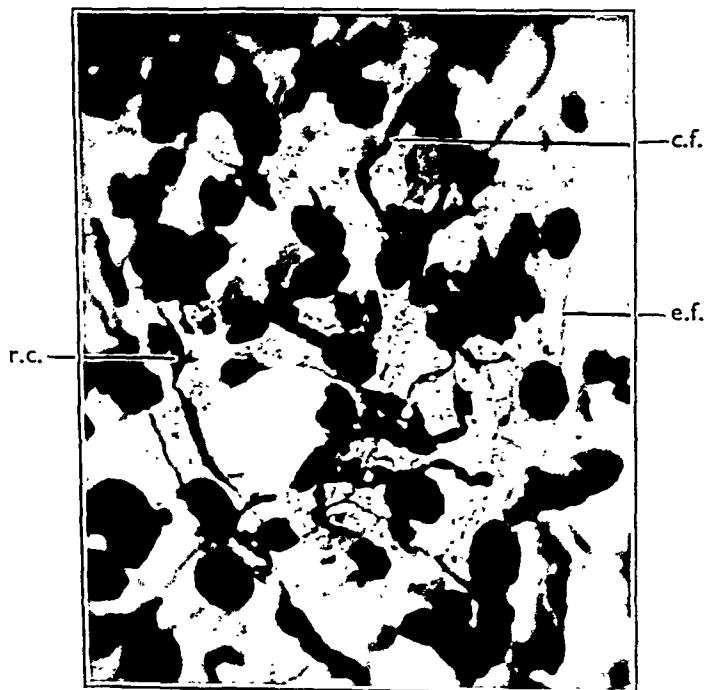
Tobacco-smoker's Pulmogram

Fig. 7, a specimen from a heavy smoker (60 cigarettes per day) otherwise normal, shows a remarkably increased number of all the cellular elements. Although there are certain parts in the film containing a lesser number of the cells, yet the greater part of the smear has the character reproduced in the photomicrograph. Giant cells which are of common occurrence in such cases are also present.

A detailed analysis of the tobacco-smoker's pulmogram under high magnification is seen in Fig. 9 and makes it clear that the majority of the cells are macrophages and in this case might be called " tobacco cells." They are loaded with particles of various sizes and shapes of which some are black and some are deep blue ; the black granules are the carbon particles which are not stained at all, and the granules staining deep blue might be partly carbonized tobacco or paper particles inhaled while smoking. Apart from the macrophages loaded with granules, there are quite numerous macrophages almost entirely free from any particles, and this may be regarded as a sign of local irritation by smoking. The nucleated alveolar epithelial cells are also much more numerous than in non-smoker's pulmogram.

The conclusions to be drawn from a smoker's pulmogram are: (1) their lungs are " infiltrated " with macrophages with increased production of giant cells ; (2) there is an increased shedding of the alveolar epithelium. The degree of these alterations depends, of course, on the daily amount of tobacco inhaled.

Comments

Aspiration lung biopsy may be used as a diagnostic procedure in various pathological conditions of the lung such as pneumoconiotic or chronic and acute inflammatory processes. The pathological alterations may be viewed on the bases of quantitative and qualitative changes of the cellular elements as well as changes of micro-histochemical analysis ; this may be of particular value in the various types of pneumoconiosis, which may offer diagnostic difficulties clinically.

The most common complication of the aspiration lung biopsy is pneumothorax which in the present series occurred in three cases in a very benign form ; pneumothorax per se, if not of very great· degree, should not be regarded as serious. On the contrary it might even have a certain beneficial effect in inflammatory lung conditions. In non-inflammatory cases it is usually a harmless event generally escaping notice.

The few accompanying photomicrographs give some idea of the value of this method in clinical diagnosis. Further investigation is needed to elicit its real value in the various pathological conditions of the lungs.

Summary

1. A cytological study is made of the aspiration lung biopsy in normal and pathological conditions.
2. The method of the biopsy is described.
3. Suggestions are made for further investigations along these lines.

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THE INFLUENCE OF ANTICOAGULANTS ON FIBRIN NETWORK FORMATION

BY

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In recent years great efforts have been made to identify the stages of the process of coagulation. The object of this study was to demonstrate the effect, if any, of certain anticoagulants on the production of the fibrin network in a thrombus.

Technique

In general the method described by Fonio and Schwenderer (1942) was used. Ten ml. of blood taken from a fasting patient with a dry syringe was divided equally and put into two paraffined test tubes which had been kept in a refrigerator. Thereafter the blood was centrifuged for 2 minutes at 3,000 revolutions per minute. The plasma thus obtained was transferred in a dry, carefully cleaned pipette to a slide which had been sprinkled lightly with particles of burned kaolin powder under observation by a dark-field microscope. The kaolin particles were added to provide crystallization centres and focusing points. The preparation was then covered with a polished cover-slip and observed by means of an oil-immersion objective in which an iris diaphragm was incorporated.

Two test-tubes were used because it had been found that the second sample frequently clotted spontaneously during centrifuging, a behaviour which could possibly be explained by the presence of bubbles of froth formed in expressing the blood from the syringe : this factor might well influence blood-clotting. The microscope was focused either on suspended thrombocytes or better on the particles of kaolin. The total time, from the drawing of blood until the focusing on the preparation, was approximately 7 minutes.

Normal Findings

During the next 7 minutes the transformation of fibrinogen into fibrin occurred. The first fibrin needle suddenly formed on a crystallization centre (thrombocyte or kaolin particle) ; subsequently other crystals grew from it. In a short time fibrin crystals appeared on the other centres as well : and within 5 minutes the whole field was covered with a fibrin network, single " needles " being of varying lengths and forming an irregular reticulum. The development of the fibrin network was photographed by a " Mifilmca Leitz " camera, the exposure being 20 seconds with 23° Scheiner cinema film.

The fibrin network was observed for several hours, some of the specimens being examined for as long as 24 hours. No considerable changes in the network were detected. Only occasionally, after 1 to 6 hours, haemochronia appeared in the interstices, showing a lively Brownian movement. These haemochronia have been seen even in the plasma of patients who had fasted for more than 12 hours, and they are therefore most likely an expression of the normal lipaemia.

Observations on Changes Produced by Anticoagulants

In cases with a lowered prothrombin level produced by the coumarin compound " pelentan " (Kubík and Reiniš, 1948), a difference in fibrin formation was noted the intensity of which was proportional to the degree of the fall in prothrombin level. The onset of the formation of the first fibrin needles was not delayed in comparison with the normal, but the development of the fibrin network proceeded more slowly. Delicate, long fibrin crystals were formed, producing an " asthenic " form of the network. The picture observed within 5 minutes in normal plasma was not reached until after several hours. A similar picture has been seen in icteric plasma, in which the prothrombin level has been lowered by hepatic damage or by retention of bile.

The effect of heparin was also observed. If added to blood *in vitro*, this substance interferes with coagulation for an unlimited time : even after seven days fibrin needles could not be detected. One ml. of heparin (50 mg.) was given intra-

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FIG. 1.—Normal plasma. First fibrin needles suddenly appearing on a crystallization centre. (Time approximately 7 minutes after bleeding.) (Magnification of all pictures : microscopic, × 750 ; photographic, × 27.)
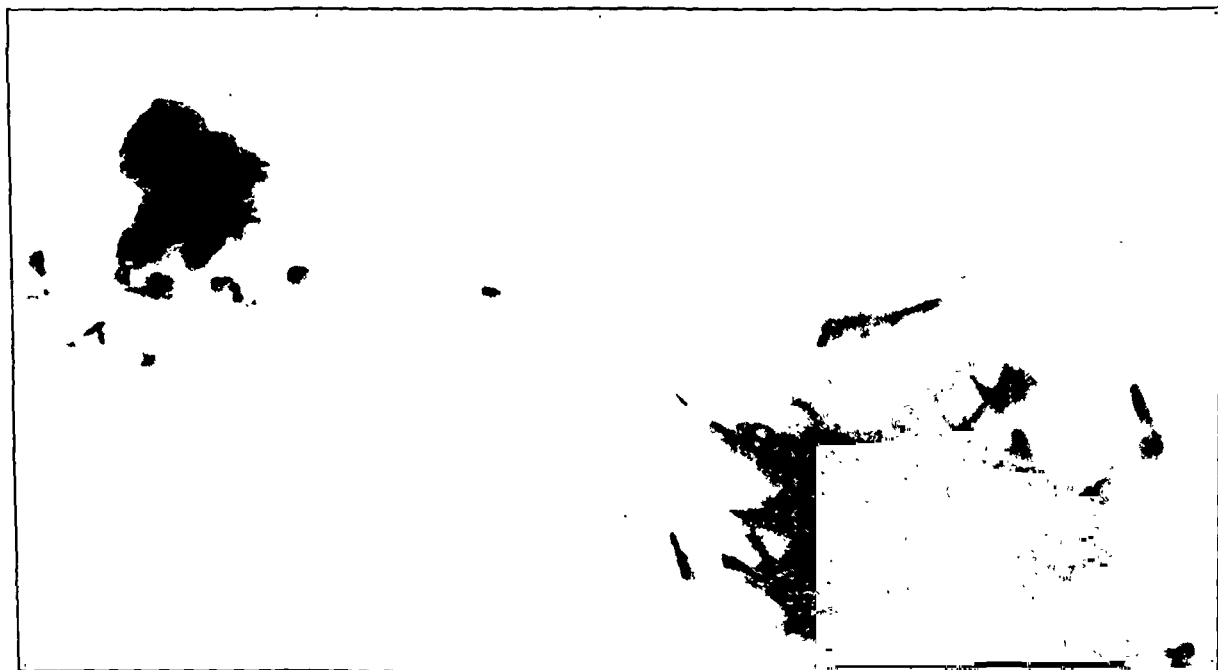
FIG. 2.—Normal plasma. Fibrin crystals forming on secondary centres in subsequent seconds.
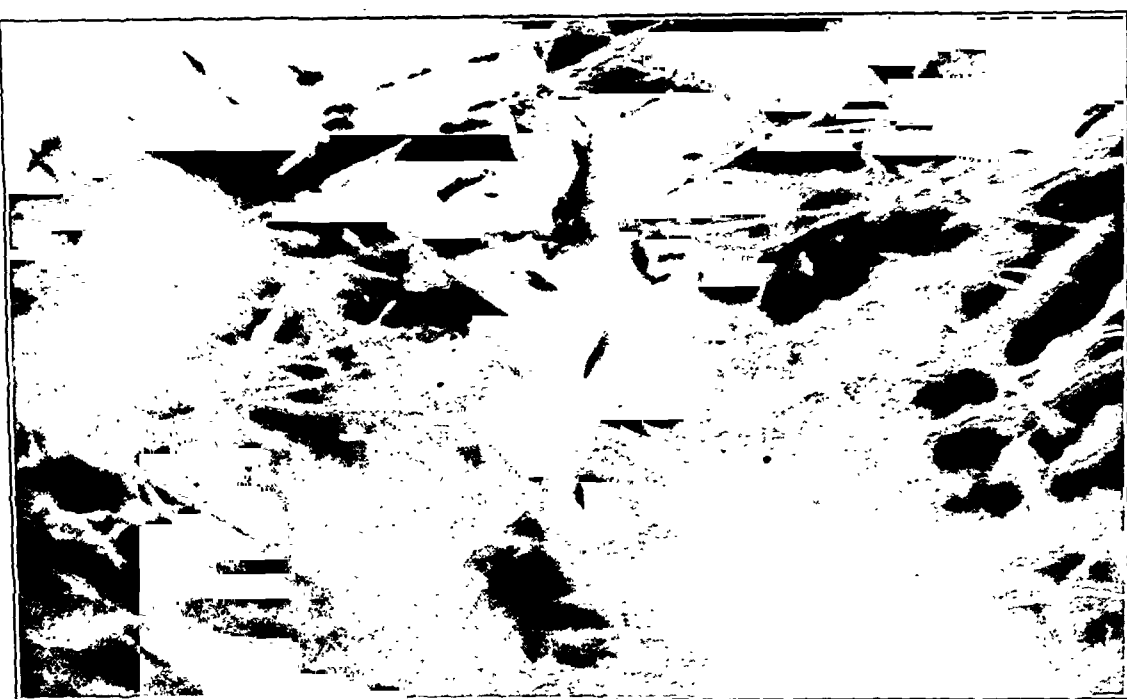
FIG. 3.—Normal plasma. Definite fibrin network made by large needles of varying lengths. (Time 5 to 7 minutes after formation of first crystals.)

FIG. 4.—Plasma of a patient treated with a coumarin compound. Prothrombin level 25 per cent of normal. Only delicate, long fibrin crystals are seen, which developed after considerable delay. (Time 1 hour after bleeding.)
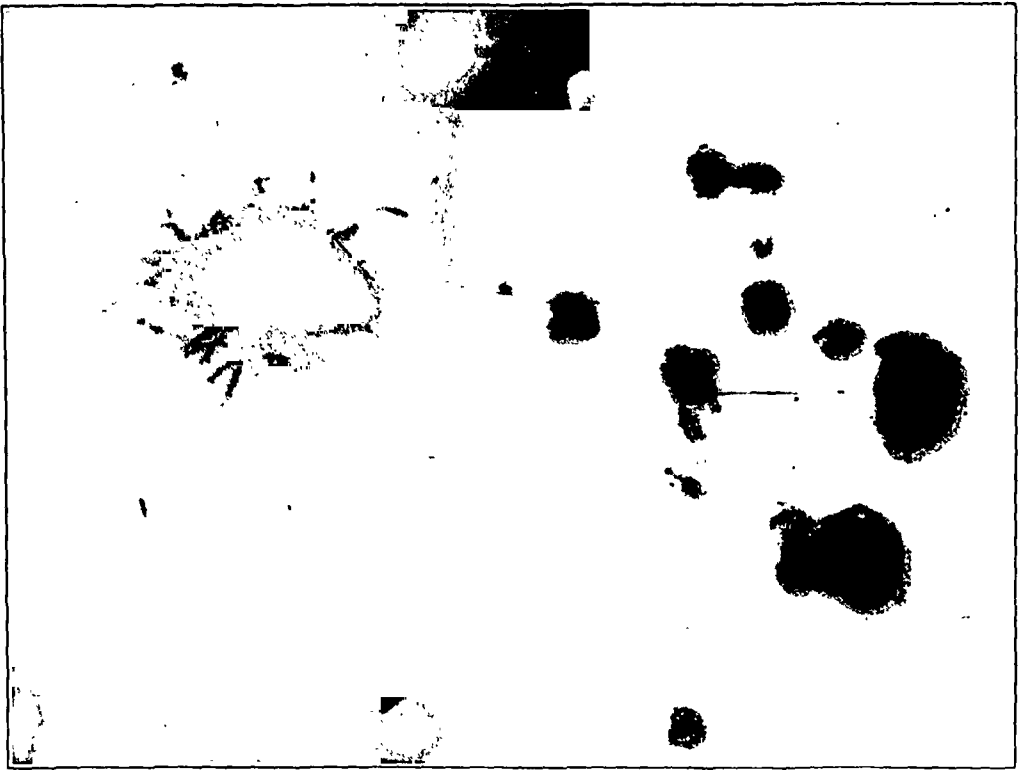
FIG. 5.—Plasma of a patient 3 hours after injection of 50 mg. of heparin. First phase of fibrin crystal formation. (Time 7 to 9 minutes after bleeding.)
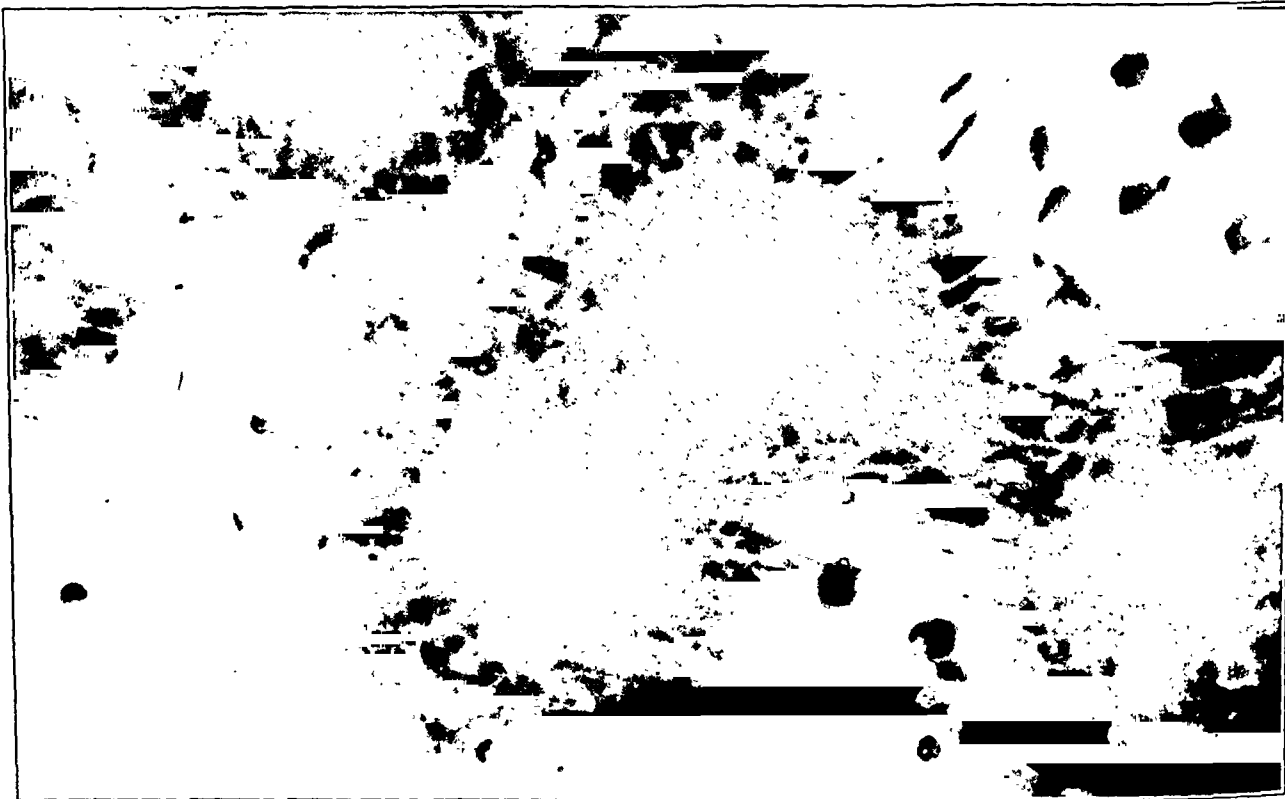
FIG. 6.—Plasma of a patient 3 hours after injection of 50 mg. of heparin. Delicate crystals heaped up around the crystallization points. Picture of definite network. (Time approximately 15 minutes after bleeding.)

venously, and the first sample was taken 20 to 30 minutes after the injection. No crystals were seen to form in these specimens when they were examined immediately ; the blood in the test-tube remained unclotted. Twenty-four hours later, however, if a specimen were taken from this test-tube, the fibrin network formed within the normal time. It appears that passage through the body so alters heparin that its efficacy becomes exhausted in the blood in the test-tube. This late network has different morphological properties which are the same as those seen in blood taken 3 hours after a previous heparin injection. In both, the time of appearance of fibrin crystals formation is normal (7 minutes) after the slide has been prepared, or approximately 15 minutes after the blood has been withdrawn. Although the velocity of development of the network is apparently about the same as in normal plasma, the crystals are more delicate, and the mesh of the network is smaller, with characteristic " hedgehog " formations heaped up around the centres of crystallization.

Because it is extremely difficult to reproduce *in vitro* all the biochemical and mechanical factors which take part in this reaction, exact conclusions cannot be drawn. Some artificial conditions are produced in the preparation of the specimens which differ from intravascular conditions. Nevertheless, it is felt that in this way a survey was obtained of the morphology of this phase of the process of coagulation, and the findings provide some idea of the structure of a thrombus and how this is influenced by anticoagulants.

Summary

By means of a dark-field technique the formation of fibrin network has been observed. Findings under normal conditions, and changes caused by administration of heparin and of a coumarin compound, are described.

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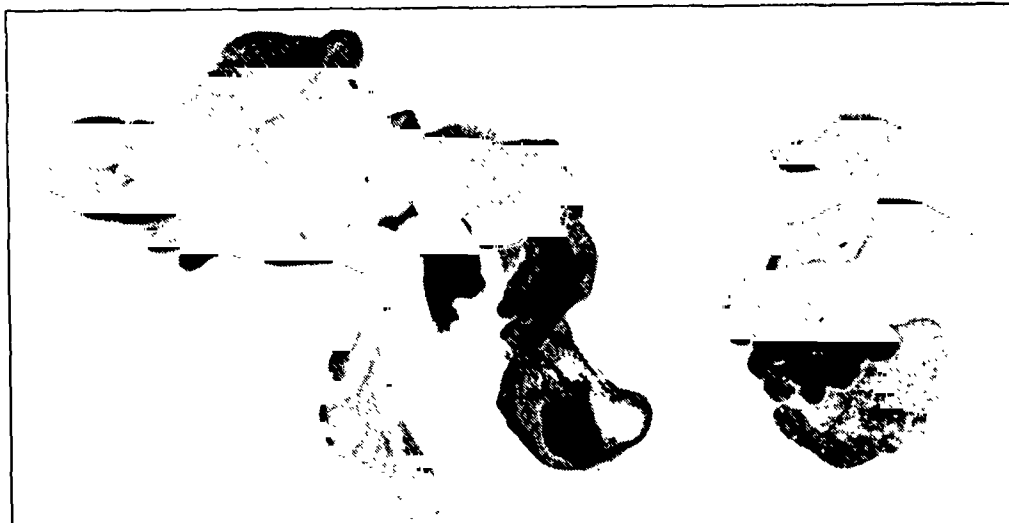
FIG. 1.—Portions of the parasite after fixation in formaldehyde. (Scale 4·5 mm. = 1 inch.)

Above, left:

FIG. 2.—Compressed scolex unstained, showing suckers and hooklets. (× 100.)

Above, right:

FIG. 3.—Section of parasite. Haematoxylin and eosin. (× 24.5.)

FIG. 4.—Same as Fig. 3, showing details of scolex. (× 145.)

INTRA-MEDULLARY CYST OF THE SPINAL CORD DUE TO THE CESTODE *MULTICEPS MULTICEPS* IN THE COENURUS STAGE

REPORT OF A CASE

BY

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The cestode *Multiceps multiceps* is well known as a cause of intracerebral cysts in sheep, but there are only three reports of similar human infestation certainly due to this particular parasite (Brumpt, 1936 ; Clapham, 1941 ; Cluver, quoted by Craig and Faust, 1943). The subcutaneous tissues in man have been more frequently involved ; there are some eight reports from various continents. The case now to be detailed is the first in which the spinal cord has been affected. A parallel case of hydatid cyst in the spinal canal has been published by Rogers and Tudhope (1938) ; the measurements of the hooklets in their case and the present case establish the different species involved.

Case Report

A girl aged 14 was admitted to the Brentwood Annexe of the London Hospital under Mr. Northfield on Feb. 15, 1947. She was suffering from spastic paraplegia of acute onset at the level of the sixth thoracic segment ; her earliest symptom, " pins and needles in her legs," appeared and increased in intensity only four weeks before motor and sensory paralysis became complete. Double incontinence followed three weeks later, but she never complained of any pain.

Laminectomy and exploration of the cord were undertaken on Feb. 19, 1947, on a clinical diagnosis of intra-medullary tumour, the spines and laminae of the fourth to the ninth thoracic vertebrae being removed. The dura was tightly stretched over a swelling of the cord, the surface of which was irregular and had a patchy bluish discoloration. A midline incision was made into the posterior surface of the cord at about the fifth and sixth thoracic segments, and a thin-walled cyst, which presented itself spontaneously through the opening, was easily and apparently completely delivered by gentle traction ; it had no visible attachment to the cord. The paralysis did not improve, and a second operation a month later, exposing the whole length of the cord from the fourth to the ninth thoracic segments, showed no further cysts and no

visible cause for the continuing paralysis. During ten months since this second operation there has been very slight sensory recovery but no motor recovery, and increasingly severe and painful flexor spasms have required relief by cordotomy.

Special investigations.—Four examinations of the blood showed no eosinophilia, 450 eosinophils or fewer being counted in 6,100 to 9,200 white cells.

The Casoni test was negative, but was not tried until some weeks after the removal of the cyst.

In already reported cases there is no record of blood examinations. Casoni tests in two patients with subcutaneous coenuri—described as *Multiceps serialis*—were also negative.

Further inquiry about the patient revealed that she had never been abroad, but had been evacuated during the war to Glamorgan, where the worm is common enough, and here she had been in close contact with the sheepdogs at a farm. She returned to Kent a year before the onset of her illness ; there had been possible contact with a young puppy—an unlikely carrier of the definitive stage—but no close or frequent contact with other animals.

Pathological examination.—The cyst (Fig. 1) was received already fixed in formol-saline, and consisted of a chain of vesicles attached to each other in a twisted and tangled group : individual vesicles were up to 0.6 cm. in diameter and had filmy transparent grey walls with white nodules (0.1 cm. in diameter), varying in number from two or three up to thirty, on the inner surface. These nodules were usually in small groups (up to eighteen) but also occurred singly.

When a nodule was compressed under a cover-slip and examined under low magnification it showed the four suckers and double row of hooklets characteristic of a Taeniid cestode (Fig. 2) : the multiple scolices in each vesicle identified the type of cyst as a coenurus. Professor Buckley, of the London School of Hygiene and Tropical Medicine, very kindly completed the identification by measurement of the hooklets as *Multiceps multiceps*, the sheep gid-worm. Unfortunately, as all the material was fixed in formalin, this could not be confirmed by identification of the

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definitive stage by passage through a puppy. Further helminthological details of this case have since been published by Crusz (1948).

Histology.—A group of scolices and part of the cyst wall were embedded in paraffin and sections were stained with haematoxylin and eosin, iron haematoxylin and van Gieson, and Mallory's phosphotungstic acid haematoxylin.

The cyst separated cleanly from the tissues of the spinal cord, no part of which was present in sections. The outer wall consisted of homogeneous eosinophil material 50 μ thick, thrown up into very numerous rounded wrinkles 20 to 30 μ in diameter; the fibres composing the wall formed a coarse reticulum. On the inner surface was a spongy parenchyma with numerous small rounded nuclei, often in groups; the confluence of vacuoles of this parenchyma formed the cyst cavity. The scolices were developed in outgrowths of the parenchyma by invagination from the cyst; this invagination appeared in section as irregular cleft lined by the cuticle of the scolex (Fig. 3). This was a smooth, uniform, fibreless membrane (10 μ thick) much folded, which stained a deeper and more purple tint in haematoxylin and eosin than the outer cyst wall. The suckers and hooklets are easily made out in the apex of the invaginations (Fig. 4).

For further study of the reactions of the host to the cyst I am indebted to Professor L. P. Garrod for the loan of a slide, stained with haematoxylin and eosin, of the cyst in Clapham's case, and to Professor Buckley for a sheep brain containing a coenurus.

The human material shows separation of the cyst wall from the brain by a space of 250 to 500 μ. This is clearly a natural line of cleavage of which the surgeons in my case took advantage. From the border of this gap, four zones can be made out in the brain before normal nervous tissue is reached, at the depth of some 3 mm. from the cyst wall.

1. The first zone (100 μ deep) is extensively necrotic and is composed of angular, sometimes multinucleated cells, orientated at right angles to the cyst and separated by numerous clefts and spaces. Preservation is too poor to determine the character of the cells but some undoubtedly are macrophages.

2. The second, less well defined zone (100 to 300 μ) consists of delicate fibrous tissue, the fibres lying parallel to the surface.

3. The main zone of cellular infiltration succeeds this with an indefinite boundary and is up to 700 μ thick, though averaging only 200 μ. The infiltration, denser round small blood vessels, is mainly of small lymphocytes with a few plasma cells. Hyaline, probably collagenous, areas with

spindle cells are also present, and a larger artery is undergoing purulent inflammation and necrosis.

4. In the nervous tissue proper there is an increase of large plump astrocytes which are arranged in such a way as to suggest severe compression; this zone is oedematous in places and up to 400 μ wide. The adjacent cortex shows chromatolysis and dropsical degeneration of a large number of pyramidal cells.

Collectively these observations indicate a chronic granulomatous inflammation forming a zone at least 3 mm. deep around the parasite. In the spinal cord a corresponding zone would be of greater functional importance than in the brain, and the clinical course of the case and the non-recovery after the removal of the cyst are thus easily explained.

In material from the sheep there is again a very obvious line of cleavage between the cyst wall and the reaction zone of giant cells, fibrin deposition, and necrosis in the adjacent tissue. This zone is a great deal wider than in the human, and the giant cells are more numerous and much larger. There is rather less small round-cell infiltration, and no necrosis in the outer adjacent zone. Traces of collagen are present in the edges of the more intense reaction.

Although, therefore, there is a very intense reaction to the presence of the cyst, this latter does not become incorporated in the host tissues, and can be expected to "shell" out on surgical manipulation; and, unless compression has already resulted in severe damage to important nervous tissues, operative treatment should have a very good chance of success.

Literature

Crusz (1948) has reviewed from the helminthological point of view the cases of coenurosis reported in man, including the present case, and both he and Clapham (1942) consider that the three types given specific rank by previous authors (for example Faust) are not true species but rather physiological strains or immature stages of the single species *M. multiceps*. In Brumpt's report a Parisian locksmith, aged 40, presented with fits and aphasia and was found at autopsy eight months later to have two cysts, one in the posterior horn of the left lateral ventricle and one in the left angular gyrus; the latter was degenerate and contained only hooklets, but 75 scolices were found in the ventricular cyst.

No details of the case reported by Cluver in the lateral ventricle of an African are available: the original reference (not given by Faust) cannot be traced.

For my knowledge of the clinical details of Clapham's case I am indebted to Dr. D. H. Fulton, pathologist to Peterborough Hospital. A man of 40 was found in coma in his bedroom and died 20 hours later, his relatives apparently mistaking his illness for acoholism. He had served in the Navy from the age of 18 to 32 and had been well apart from an attack of meningococcal meningitis at the age of 18. At the age of 36 he had the first of increasingly frequent and severe sudden headaches, which lasted up to 24 hours. He had no fits or paralyses. At autopsy the right cerebral hemisphere was larger than the left; on section a thin-walled cyst 5 cm. in diameter by 8 cm. from front to back was found above and lateral to the right lateral ventricle; its inner wall was studded with white, raised areas, and it contained clear fluid. The left hemisphere and the cerebellum were normal. A massive haemorrhage had occurred in the pons and medulla and was the cause of death. The thoracic and abdominal organs were normal. He had died with arms and legs extended and with hands tightly clenched. His headaches with their paroxysmal onset resemble those occurring with colloid cyst of the third ventricle or the headache of acute internal hydrocephalus, and a terminal pontine or mid-brain haemorrhage is a not uncommon sequel to increased supratentorial pressure. The infection was probably acquired in this country rather than during his Naval service. This is suggested both by the lapse of four years between leaving the service and the onset of the headaches, which may be taken as marking the onset of the cerebral lesion, and by the life history of the parasite. This develops to full maturity in the sheep in about eight months (Neveu-Lemaire, 1936); it is then about 3 to 5 cm. in diameter and causes softening of the overlying skull. It occurs all over the world where sheep-raising is carried on, the definitive stage being passed in the intestine of the dog or wolf and the intermediate cyst (*Coenurus*) stage in the central nervous system of herbivores. In this country it is fairly well known in the Welsh sheep farms; but the Chief Inspector of Abattoirs in Cardiff has only seen two cases in twenty years, since the sheep commonly die on the farms. In some cases the shepherds remove the cysts with their penknives, locating them by the softening of the skull. The name " gid-worm " is derived from the staggering of the sheep when affected by the parasite.

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THE ALBUMIN/GLOBULIN RATIO: A TECHNICAL STUDY

BY

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In 1885–6 Kauder observed that globulin was precipitated from serum when the concentration of ammonium sulphate reached 24 to 29 per cent of full saturation and that its precipitation was complete when the concentration reached 36 to 46 per cent of complete saturation. Thereafter no further precipitation took place until the concentration reached 64 per cent of full saturation, when albumin started to precipitate, its precipitation being complete at 90 per cent saturation. The simple qualitative differentiation of albumin from the globulin by their behaviour in the presence of ammonium sulphate is based on this and like observations.

Howe (1921), investigating the properties of sodium sulphate as a precipitant, demonstrated three zones in which variations of the concentration of the sodium sulphate did not produce any significant change in the amount of protein precipitated. These lay between 13.5 and 14.5 per cent, between 16.4 and 17.4 per cent, and between 21 and 22 per cent, using anhydrous sodium sulphate. This last concentration precipitated approximately the same amount of protein as did half saturation with ammonium sulphate. It was assumed to represent the point at which the globulins had been completely precipitated from solution. The use of the sodium salt for the precipitation enabled the estimation of the residual soluble protein to be carried out by the Kjeldahl technique, and soon the method was adopted widely as a routine procedure in the estimation of the " albumin/globulin ratio."

The application of the more delicate physico-chemical techniques to the study of the serum proteins, and in particular the accumulation of data derived from analyses made by the electrophoretic technique of Tiselius, makes it desirable to check how far the common practical routine methods yield results comparable with those obtained by the more delicate physico-chemical techniques.

Dole (1944) demonstrated clearly that the albumin-globulin ratio of normal plasma calculated from electrophoretic data was constantly at variance with that obtained by the Howe fractionation technique. Subsequently one of the writers, in studying sera from diseased persons, noted not only that there was divergence but that the discrepancy was on occasion such as to suggest that the cruder technique was yielding meaningless results in terms of the electrophoretic data. Other workers (Pillemer and Hutchinson, 1945; Majoor, 1947; Milne, 1947) have been exercised by the same problem.

If the routine procedure of reporting the albumin/globulin ratio was to retain any meaning at all it seemed highly desirable to adopt a procedure which, while simple enough for routine procedures in a busy laboratory, gave results which bore some reasonable relationship to the figures obtained by more elaborate methods of analysis. With this end in view some of the common laboratory techniques of separation and modifications of them were examined.

Materials

The sera were drawn from patients with a wide variety of clinical conditions in whom qualitative and quantitative disturbances of circulating proteins were anticipated.

Methods

The following single-stage methods of protein fractionation were examined:

1. (*a*) Precipitation of the globulin fraction using sodium sulphate according to the technique of Howe; (*b*) precipitation of the globulin fraction using 1.83 molar sodium sulphate (26 per cent sodium sulphate) at 37° C. (Majoor, 1947).

2. Precipitation with sodium sulphite according to the technique originally outlined by Campbell and Hanna (1937).

3. Precipitation with magnesium sulphate following the technique outlined by Popják and McCarthy (1946).

4. Precipitation by methanol in an acetate buffer according to the technique of Pillemer and Hutchinson (1945).

The conditions of precipitation are shown in Table I.

Robinson and others (1937) have observed that repeated filtration results in adsorption of protein

TABLE I

CONDITIONS FOR PROTEIN PRECIPITATION

	pH	Conc. g./L	Approx. final molar conc. before ppt.	Alcohol conc.	Temp. °C.	Range of protein conc. before ppt. (in g. per 100 ml.)	Time allowed for complete precipitation (hours)
Sodium sulphate Na_2SO_4	6.4 ± 0.04	225	1.53	nil	37	0.38–0.14	16
Sodium sulphate Na_2SO_4	6.5 ± 0.04	260	1.77	nil	37	0.38–0.14	16
Sodium sulphite $Na_2SO_3 7H_2O$	9.6 ± 0.04	420	1.59	nil	20	0.58–0.210	$\frac{1}{6}$
Magnesium sulphate saturated sol.	7.0 ± 0.2	360	3.00	nil	20	1.45–0.53	24
Methyl alcohol in sodium acetate buffer	6.7 ± 0.05		0.06*	42.5%	0–2	2.3–0.85	$\frac{1}{4}$

* Approximate molarity of acetate buffer.

from solution on the filter paper. Filtrations, therefore, were carried out once only. If the filtrate was not clear it was rejected. The nitrogen estimations were carried out by the micro-Kjeldahl technique, which was standardized by the estimation of the nitrogen content of weighed amounts of crystalline bovine albumin.

The protein values calculated from the nitrogen estimations were correlated with an electrophoretic analysis of the same sample of serum after dialysis against a phosphate buffer and run at pH 8.0 in a 0.2 M-sodium phosphate buffer, the protein concentration being adjusted to 2.0 g. per 100 ml. buffer.

The pH of the various precipitating solutions was estimated using the standard glass electrode, measurements being made before and after precipitation of the globulin fraction. The pH of the solutions is shown in Table I.

Results

The results of the various analyses are compounded in Table II. The electrophoretic analyses of the soluble proteins after precipitation by the Howe technique (22.5 per cent sodium sulphate) has been shown by Perlmann to consist of appreciable amounts of α and β globulins. By increasing the concentration of sodium sulphate to 26 per cent a fractionation was obtained which approximated much more closely to the albumin/globulin ratio obtained by electrophoretic analysis (compare columns b, g, and i in Table II). Electrophoretic analysis of the soluble

TABLE II

TABLE OF ANALYSIS

No.	Serum	Total protein by micro-Kjeldahl g./100 ml. (a)	Electrophoretic analysis fractions as g./100 ml.					After precipitation with Na₂SO₄		After precipitation with MeOH with acetate	After precipitation with Na₂SO₄	After precipitation with MgSO₄
			Alb. (b)	Globulins				26% (g)	22.5% (h)	(i)	(j)	(k)
				α₁ (c)	α₂ (d)	β (e)	γ (f)					
1	E (ter)	6.20	1.27	0.23	0.39	1.01	3.3	1.2	2.15	1.25	2.4	1.9
2	M₂	7.85	2.85	0.7	1.9	1.4	1.0	2.8	3.4	2.9	4.0	3.35
3	E₂	7.6	2.8	0.55		1.5	2.6	2.7	3.2	2.7	2.9	3.1
4	Br₁	5.1	2.3	0.92		1.02	0.82	2.1	2.55	2.35	2.8	2.35
5	R₁₁	5.1	2.55	1.27		0.76	0.51	2.3	2.5	2.5	2.8	2.6
6	P	4.2	1.85	0.3	0.34	0.82	0.8	1.8	2.3	1.9	2.4	
7	S	4.4	0.97	1.8	0.48	0.52	0.67	0.96	2.7	1.0	2.8	
8	E₁	8.8	2.8	0.5		1.8	3.6	2.7	3.2	2.7	3.4	
9	II₂	6.8	1.7	1.36	1.63	1.5	0.60	1.8	3.2	1.75	3.0	
10	Cr	7.2	3.2	0.22	1.2	1.73	0.72	3.2	4.0	3.4	3.7	
11	II₂	6.0	2.55	0.5	0.75	0.95	1.2	2.6	2.9	2.7	3.0	
12	B₁	11.6	1.21	—	0.98	—	9.35	1.2	2.1	1.25	1.7	
13	M₁	8.5	4.15	0.76	0.85	1.53	1.02	4.2	5.1	4.4	4.8	
14	Cl	7.6	3.6	0.4	0.7	1.75	1.2	3.6	4.3	3.55		
15	B₂	8.5	1.06	—	0.9	—	6.5	1.2	2.0	1.03		
		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)

portion in fact demonstrated that a small but appreciable portion of α globulin remained in solution when precipitation was complete. This is of fundamental importance when considering properties attributable to this soluble fraction. The soluble proteins obtained by the alcohol acetate fractionation of Pillemer were also examined electrophoretically, and it will be seen from Table II, column i, that they, too, gave results approximating well with the electrophoretic analysis.

Discussion

Because the amount of serum from individual patients was limited, the majority of estimations was concentrated on two techniques which in our hands seemed to give results corresponding most consistently with electrophoretic data.

It will be seen that both the classical Howe technique and the technique of Campbell and Hanna, using sodium sulphite, are at considerable variance with the albumin/globulin ratio deduced from electrophoretic analysis. A further objection to the use of sodium sulphite is that the pH of the precipitating solution lies so far to the alkaline side of neutrality that there is risk of denaturation of some of the proteins under examination and of alteration in their solubility. Though Cohn and others (1946) have shown that if the temperature of the mixture is kept low the use of the organic solvents is permissible in affecting separation, they stress the caution with which such solvents must be used. Our examination of the soluble fraction from the acetate methanol separation suggested that on occasion some slight degree of denaturation may occur. We do not believe that this is frequent or extensive if care is taken in maintaining a low temperature during precipitation. We mention it to stress the care that must be exercised

when employing organic solvents in separation. Apart from this defect the method advocated by Pillemer was clean, rapid, and convenient. That, and the separation by 26 per cent Na_2SO_4 along the lines outlined here and as laid down by Majoor (1947), have in our hands offered the most convenient and most accurate routine procedures for the estimation of the albumin/globulin ratio in a clinical laboratory.

Summary

Salting-out methods for determination of serum proteins have been compared with the figures obtained by electrophoretic methods.

By the use of 26 per cent sodium sulphate or methanol precipitation, close agreement with the electrophoretic values was obtained.

Twenty-two per cent sodium sulphate, sodium sulphite, or magnesium sulphate gave results which differed significantly from the electrophoretic values.

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MEASUREMENT OF THE GLOMERULAR FILTRATION RATE AND THE EFFECTIVE RENAL PLASMA FLOW USING SODIUM THIOSULPHATE AND p-AMINO-HIPPURIC ACID

BY

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The clearance methods for the measurement of the glomerular filtration rate, the effective renal plasma flow, and the maximum tubular excretory capacity, which were originally developed by Professor Homer Smith and his associates, have found increased clinical application in America. These tests yield information of fundamental physiological significance, but they cannot be undertaken lightly because they are time-consuming both for patient and doctor. Many of the methods previously employed made use of a continuous drip infusion; either inulin or mannitol was employed to measure the glomerular filtration rate, and diodrast or p-amino-hippuric acid to measure the effective renal plasma flow and the maximum tubular excretory capacity. Recent modifications include the use of special infusion pumps which deliver at a constant rate so that equilibrium between injection and excretion can be attained (Earle and Berliner, 1946) and the adoption of a single-injection technique (Landowne and Alving, 1946; Josephson, 1947; and Newman and others, 1946).

Gilman and others (1946), while examining the mechanism of sodium thiosulphate excretion in the dog, found that the clearances of sodium thiosulphate and creatinine were identical. They assumed that sodium thiosulphate clearance was a measure of the glomerular filtration rate in the dog. Subsequently they measured simultaneously, in man, the clearances of sodium thiosulphate and inulin. Single large doses of the two substances were given intravenously (10 g. of inulin in 10 per

cent solution followed by 1 g. of sodium thiosulphate per 10 pounds of body weight). They found that the ratio of inulin to sodium thiosulphate excretion, as measured on declining blood concentrations, averaged 0.99, with a standard deviation of 0.08. These results were based on seventy-three observations; they assumed that sodium thiosulphate clearance was a measure of the glomerular filtration rate in man.

This single-injection method, while eliminating the technical disadvantages of a continuous infusion, does not permit observations to be made over a long period because the plasma concentration soon falls below the level at which it can be accurately estimated. It does not permit of simultaneous measurement of the effective renal plasma flow by p-amino-hippuric acid because the concentration of this substance in the plasma must be kept below 4 mg./100 ml. in order to ensure complete clearance in a single passage through the kidney (Goldring and Chasis, 1944). Lastly, the single injection of a large amount of sodium thiosulphate is liable to produce toxic effects; seven of the 26 patients studied by Gilman suffered nausea and vomiting.

The present work is an attempt to establish a reasonably simple method of measuring the glomerular filtration rate, using non-toxic doses of sodium thiosulphate, administered by continuous infusion. In ten normal male medical students the clearances of sodium thiosulphate and inulin have been measured consecutively; the drugs cannot be administered simultaneously in the same drip

TABLE I

COMPARISON OF INULIN AND SODIUM THIOSULPHATE CLEARANCES IN TEN NORMAL MALES

Case no.	Age	Height and weight	Inulin clearance ml. per min.	Thiosulphate clearance ml. per min.	Mean inulin clearance corrected to 1.73 sq. m.	Mean thiosulphate clearance corrected to 1.73 sq. m.
1	20	5 ft. 6 in. 10 st. 1 lb.	a. 168 b. 165	164 165	166.5	164.5
2	32	6 ft. 2 in. 11 st. 8 lb.	a. 132 b. 138	121 113	119	103
3	19	5 ft. 10 in. 11 st.	a. 204 b. 206	208 170	190	176
4	19	6 ft. 1 in. 11 st. 12 lb.	a. 154 b. 154	160 143	134	131
5	26	5 ft. 8 in. 12 st.	a. 148 b. 148	145 156	135	137
6	18	5 ft. 9½ in. 11 st. 1 lb.	a. 169 b. 191	177 178	168	165
7	20	5 ft. 9 in. 10 st. 6 lb.	a. 113 b. 104	113 117	104	111
8	25	5 ft. 10½ in. 10 st. 8 lb.	a. 144 b. 140	142 136	133	131
9	31	5 ft. 10 in. 9 st. 13 lb.	a. 158 b. 164	165 154	158	157
10	34	5 ft. 7 in. 10 st. 3 lb.	a. 166 b. 166	176 180	175	164
Mean ..			—	—	144	146

are shown in Table I. The clearances were corrected to a surface area of 1.73 sq. m. using Du Bois tables. There is a close relationship between inulin and thiosulphate clearances in normal individuals ; the mean clearance for inulin was 144 ml. per minute and for thiosulphate 146 ml. per minute.

The total plasma concentration of thiosulphate was 12 to 15 mg./100 ml. and of inulin 20 to 25 mg./100 ml. With these amounts accurate determinations are possible and the end point of the thiosulphate estimation is sharply definable. Both plasma and urine "blank" determinations were made ; the mean plasma level of "thiosulphate" was about 4 mg./100 ml. and of "inulin" 2 mg./100 ml. The concentration of the urine will obviously affect such "blanks" ; the specimen should be obtained under similar circumstances to those during the clearances. The concentrations of both thiosulphate and inulin in the urine were very low and may therefore be neglected.

2. THE COMBINED TEST TO MEASURE SIMULTANEOUSLY THE GLOMERULAR FILTRATION RATE AND THE EFFECTIVE RENAL PLASMA FLOW

This was carried out in three healthy male medical students ; the results are shown in Table II.

The mean clearances for normal males given by Goldring and Chasis (1944) are 697 ml. per minute for the effective renal plasma flow and 131 ml. per minute for the glomerular filtration rate, giving a filtration fraction of 0.19. In normal individuals the range is wide but the results obtained agree closely with the American workers' larger series. The effective renal plasma flow of Case 1 was low ; this particular student was very apprehensive and we are inclined to believe his effective renal plasma flow was diminished on this account while his filtration rate remained normal.

Discussion

It was our intention to employ a simple test for the study of separate aspects of renal function, and

TABLE II

THE RESULTS OF THE COMBINED TEST TO MEASURE SIMULTANEOUSLY THE GLOMERULAR FILTRATION RATE AND THE EFFECTIVE RENAL PLASMA FLOW IN THREE NORMAL MALES

Case No.	Age	Height and weight	Collection period (min.)	Urine vol. (ml.)	Sodium p-amino-hippurate					Sodium thiosulphate					Filtration fraction
					Urine conc. (mg./100 ml.)	Mg./min. excreted	Plasma conc. (mg./100 ml.)	Clearance (ml./min.)	Clearance ml./min. corrected to 1.73 sq. m.	Urine conc. (mg./100 ml.)	Mg./min. excreted	Plasma conc. (mg./100 ml.)	Clearance (ml./min.)	Clearance ml./min. corrected to 1.73 sq. m.	
1	19	5 ft. 8 in. 9 st. 10 lb.	20 20 20	88 56 55	406 530 520	17.8 14.9 14.4	3.17 2.97 2.88	560 500 500 — 520	525	325 495 465	14.25 13.75 12.80	10.4 9.5 9.1	137 145 141 — 141	142	0.27
2	22	6 ft. 2 in. 11 st. 6 lb.	17 19 20	66 100 200	446 364 240	17.4 19.4 24.0	2.15 2.03 2.47	817 962 970 — 916	795	358 274 157	13.9 14.4 15.7	8.0 7.6 8.1	174 189 194 — 186	165	0.21
3	26	5 ft. 10½ in. 11 st. 8 lb.	20 20 21	100 100 100	298 300 304	14.9 15.0 14.5	1.98 1.98 2.06	756 756 710 — 740	675	329 327 294	16.45 16.35 14.70	10.4 10.2 9.6	158 160 154 — 157	143	0.21

we hope this test may be extended for routine clinical use.

The methods already described for the accurate measurement of the glomerular filtration rate and the effective renal plasma flow have several disadvantages, especially when applied to man. Inaccurate collection of urine constitutes one of the main sources of error. In order to obtain reasonable accuracy it is necessary to perform two or three serial collections of urine, employing continuous drainage through an indwelling catheter and washing out the bladder at the end of each collection period with saline and air. The time of the collection period can, of course, be accurately measured by a stopwatch. The time devoted to the collection of blood and urine samples is considerable, but three clearances of about 20 minutes each can be completed within two hours. Apart from the necessity of catheterization, the discomfort to the patient, involving one venepuncture in one arm for the infusion, and four venepunctures in the other arm, is not excessive. None of our patients complained of undue discomfort, and none suffered toxic symptoms from sodium thiosulphate.

We conclude that the method we have outlined employing sodium thiosulphate and p-amino-hippuric acid for the measurement of the glomerular filtration rate and the effective renal plasma flow gives accurate results by means of chemical analyses which are within the scope of most laboratories.

Summary

1. A comparison of inulin and sodium thiosulphate clearances in 10 normal males, and the results of the combined test to measure simultaneously the glomerular filtration rate, and the effective renal plasma flow in three normal males are presented.

2. Using a continuous drip infusion sodium thiosulphate appears to offer a reliable means for the determination of the glomerular filtration rate; it is cheap and non-toxic, and the estimation is simple and accurate.

3. Sodium thiosulphate does not affect the excretion of sodium p-amino-hippurate.

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BACTERIAL FIBRINOLYSIN, ITS POSSIBLE THERAPEUTIC APPLICATION IN TUBERCULOUS MENINGITIS

BY

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A constant feature at autopsy in cases dying from tuberculous meningitis, whether treated with streptomycin or not, is a gelatinous exudate at the base of the brain. Frequently this is found in association with, or causing, hydrocephalus, and once the exudate has been formed it is difficult to visualize how streptomycin treatment alone could achieve its resolution. Even were streptomycin able to penetrate and sterilize the exudate the most likely end result would be its organization to fibrous tissue with an increase in the conditions leading to hydrocephalus.

The known propensity of cerebrospinal fluid from cases of tuberculous meningitis to form spider's web clots on standing which give the reactions of fibrin, combined with the fibrinous nature of the exudate, has led to the consideration of other forms of therapy in combination with streptomycin which might inhibit fibrin formation in the thecal space. Of the possible anticoagulants, heparin has received the most attention. The drawback that it forms a deposit with streptomycin may be avoided by spaced dosage or by the method of St. Hill and others (1948), in which heparin is added to streptomycin until no further deposition occurs, after which the supernatant fluid is removed by decantation. This supernatant fluid contains all the streptomycin used, and heparin may be added to it without the formation of a deposit.

Our experience of this combination has been disappointing as judged by the results seen in several patients at autopsy, and, indeed, although it is likely that heparin may prevent the deposition of further fibrin it is improbable that it would have any effect upon the fibrinous exudate, which presumably is already present when the diagnosis of tuberculous meningitis is made. For this reason some other means was sought which would have

an action more of a lytic than of a preventive nature, and eventually it was decided to investigate the possibilities of the fibrinolysin produced by some strains of bacteria.

The fibrinolysin produced by pathogenic strains of haemolytic streptococci has been examined by Tillett and Garner (1933), Lancefield and Hare (1935), Colebrook and others (1935), and by others, and a few preliminary experiments with Group A haemolytic streptococci showed that when grown in broth they produced varying amounts of a substance which was capable of lysing human fibrin clots. The variability of yield of lysin from different strains of streptococcus, and even of day-to-day cultures from the same strain, was overcome when Dr. C. Lack drew my attention to the strain of *Strep. pyogenes* known as H.64, a strain known for its abundant production of fibrinolysin. Since then only this organism has been used, and it has given consistently good yields of lysin.

Preparation of Fibrinolysin

The method of preparation of the lysin has been essentially that described by Garner and Tillett (1934), with slight modifications, and consists of adsorbing fibrinolysin from a broth culture on to aluminium hydroxide, from which it is eluted with a phosphate buffer solution.

Preparation of alumina.—50 g. of aluminium sulphate $(Al_2SO_4)_3$.18 H_2O (Willstätter and Kraut, 1924) in 150 ml. of distilled water are heated at 55° C. until dissolved. 500 ml. of a 15 per cent solution of ammonia are warmed to 55°, mixed with the alumina, and stirred vigorously for 30 minutes at 55 to 60°. The resulting precipitate is washed repeatedly with distilled water followed by decantation until no further smell of ammonia remains. Usually three or four washes are necessary. It is then washed with 100 ml. of the ammonia solution to decompose any traces of

basic sulphate. The precipitate is finally washed two or three times with distilled water and either centrifuged or filtered through a Buchner funnel using No. 1 Whatman filter paper and negative pressure. Filtration is preferred to centrifuging as the resultant paste is drier. The alumina is now ready for use.

Broth culture.—Strep. pyogenes H.64 is heavily seeded into 500 ml. of heart digest broth containing 2 per cent NaCl and 0.05 per cent glucose and incubated for eighteen hours. Much longer incubation does not give appreciably greater yields of fibrinolysin. After passage through a clarifying filter the broth is ready for adsorption.

Adsorption and elution of fibrinolysin. — The alumina paste is added to approximately 500 ml. of the broth culture and the two are agitated together while the temperature is raised to 37° C. in a waterbath. The mixture is maintained at this temperature for one hour with frequent stirring. It is then filtered through a Buchner funnel, and the deposit, on which the fibrinolysin is adsorbed, is washed by resuspension twice each in 250 ml. volumes of saline and distilled water, removing the fluid after each washing by filtration. After the last washing the paste should be as dry as possible.

The deposit is now resuspended in 100 ml. of M/10 phosphate buffer solution (35.8 g. Na_2HPO_4 in 950 ml. of distilled water, adjusted to pH 7.3 with concentrated HCl, and made up to 1,000 ml.), thoroughly stirred, and then incubated at 37° C. for thirty minutes. The alumina is then removed by filtration, and the fibrinolysin-containing filtrate is finally sterilized by means of a bacterial filter.

Although the first elution contains more lysin than do subsequent ones, second and third elutions are only slightly less potent and are well worth preparing. Later elutions are much weaker than the first few. All manipulations of the alumina are facilitated with an ordinary household egg-whisk.

Properties of Fibrinolysin

The solution prepared by the above technique consists of a somewhat variable amount of fibrinolysin in phosphate buffer. Attempts to remove the phosphate by dialysis have been unsuccessful. When dialysed with a cellophane membrane against frequent changes of distilled water there was still phosphate present after two weeks, and there was some loss of potency of the lysin. Against running tap water a deposit formed in the solution which, as the process was accompanied by total loss of lytic activity, presumably contained the fibrinolysin.

Assay of fibrinolysin.—Originally clots were prepared by the addition of an excess of calcium to plasma obtained from routine blood sedimentation rate samples of blood. In the stronger concentrations of lysin, however, the phosphate combined with the excess calcium, producing an opalescence through which the behaviour of a clot was difficult to watch. Further, it was found that plasma obtained from different patients gave results not invariably repeatable. As an outcome of these observations the following method of assay has been temporarily adopted.

From the same donor at the same time each day 0.4 ml. of blood is taken directly into 0.1 ml. of 3.8 per cent sodium citrate in a B.S.R. pipette, and the plasma so obtained is used in a dilution of 1:20 in saline. To a row of tubes each containing 0.5 ml. of diluted plasma are added one drop of thrombin solution (containing approximately 4 units of thrombin) and 0.5 ml. of serial dilutions of fibrinolysin. In the higher strengths of lysin no clot may appear, while in the greater dilutions a clot is formed within about two minutes, which is lysed in a period of time which lengthens as the lysin is more dilute. Either the end-point may be taken as the dilution of fibrinolysin which inhibits clot formation, or the tubes may be incubated at 37° C. for one hour, the end point being in that tube which has completely lysed the clot in this period. It is essential to include a control clot with saline, as occasionally the naturally occurring plasma fibrinolysin (Macfarlane and Pilling, 1946) will lyse the clot formed in it.

Not all preparations of lysin prevent visible clot formation, although the first elution from aluminium hydroxide is more likely to have this property than the later ones. For this reason the dilution capable of lysing a standard clot in one hour gives a more precise figure for comparison of potency with other batches of fibrinolysin.

Typical figures for such a titration of lysin are:

Dilution of lysin		4	8	12	16	20	24	28
Time for clot to lyse in minutes	Batch A	3	7	19	45	1		
	B	NC	NC	3	8	21	49	115

NC = No clot seen.

In these examples the end point was taken as the dilution capable of dissolving its clot in one hour, and with batch A it was 16, while with B it was 24. Batch B was therefore arbitrarily assumed to be 24/16 as potent as batch A. Adjustments of potency may be made either by dilution with distilled water or by concentration by low temperature distillation.

Preparations of fibrinolysin vary in potency within considerable limits, although obtained by exactly the same method, so that some form of

comparable assay is essential. Of successive elutions from the same alumina the fifth elution is usually about one-tenth as strong as the first.

Method of action.—A preparation of fibrinolysin was taken which permitted the initial formation of a clot in all dilutions with the standard plasma and thrombin. The time for clots to form and disappear was carefully noted. Then plasma and lysin were incubated together for 30 minutes, after which thrombin was added and the appearance and disappearance of clots were timed. Similarly thrombin and lysin were incubated, plasma was added, and clot behaviour was timed. In both these experiments the times for appearance and disappearance of the clots were the same as when plasma, thrombin, and lysin were added simultaneously. They were taken to indicate that lysin has no effect upon either thrombin or fibrinogen, and that when fibrinolysin is present in sufficient strength to prevent visible clot formation it is able to dissolve the clot as it is formed.

By varying the amounts of plasma or lysin in methods of assay, the action of the lysin can be shown to be quantitative.

Specificity of fibrinolysin.—It has been said (Tillett and Garner, 1933) that many strains of *Strep. pyogenes* produce a fibrinolysin specific for human fibrin. The fibrinolysin under discussion, however, will dissolve fibrin clots prepared from rabbit, mouse, and guinea-pig plasma, although the period is slightly longer than with human fibrin. The only failure has been with plasma from a guinea-pig with a heavy tuberculous infection.

Stability of fibrinolysin. — The remarkable stability of fibrinolysin has been emphasized by Garner and Tillett (1934). As judged by the time taken to lyse a standard clot there is no loss of potency after incubation at 37° C. for 14 days. Similarly, after heating for 45 minutes at 95° C., followed by storage in the cold, at room temperature, or at 37° C., there is no loss of activity in 14 days. Activity is completely destroyed by autoclaving for one hour at 15 lb. pressure and by acid hydrolysis.

Therapeutic Application

Several pieces of fibrinous exudate were taken from the base of the brain of cases dying of tuberculous and coliform meningitis and subjected to the action of undiluted fibrinolysin. In each case lysis of the exudate occurred in from two to seven days, depending upon the amount of exudate used. In the tuberculous cases a very fine mesh of fibrous tissue was left. As controls pieces of brain and normal meninges were used, and these remained unaltered after incubation for a month.

Clots formed on standing in the cerebrospinal fluid from many cases of tuberculous meningitis were incubated with fibrinolysin with disappointing results. In a few cases lysis took place, but in many no effect was seen, contrasting markedly with the lysis obtained with control clots prepared from plasma. In view of the good results with the post-mortem exudates, however, it was decided to investigate the clinical application of fibrinolysin.

1 ml. of a trial batch of fibrinolysin was injected into each of six normal thecas, and gave rise to cell counts of 500–800 polymorphs and protein rises of 0–10 mg. after 24 hours. This effect is similar to the pleocytosis and protein rise caused by streptomycin in a normal theca. 1 ml. of phosphate buffer without fibrinolysin was given into a normal theca and no alteration was seen in the cerebrospinal fluid after 24 hours, indicating that the irritant effect was due to the fibrinolysin and not to the phosphate. Many injections have been made intrathecally in cases of tuberculous meningitis, and usually a small increase in the existing cell count has been seen after the first three or four injections, again similar to the effect of streptomycin. In a typical case the cells rose from 200 to 280 per c.mm. 24 hours after fibrinolysin had been injected. After fibrinolysin had been given for four days no further increase in the cell count was seen, the greatest count being 390. Thereafter the cell count fell steadily, and the fibrinolysin appeared to have no adverse effect on it. The cell response was polymorph in type, and reversion to the original 95 per cent lymphocytosis was seen after two weeks.

Both the hydrochloride and calcium chloride complex of streptomycin produce a deposit with fibrinolysin in phosphate buffer, much as they do with heparin. But a mixture of the fibrinolysin solution and streptomycin sulphate remains clear, and only this salt has been used.

The variability of different preparations of fibrinolysin has been noted, and one particular yield, while not being outstandingly lytic, caused gross increases in the cerebrospinal fluid cell count of the two patients to whom it was given, one count rising from 300 to 2,000 per c.mm. in 24 hours. No sequelae to the reactions were seen, but the batch of fibrinolysin was immediately withdrawn and another substituted for it. No other batch has had this toxic effect.

As the result of trial and error the amount of fibrinolysin to be used therapeutically has been

chosen as 100 times the amount capable of lysing the standard clot in one hour. Thus, when a preparation lyses clots in a dilution of 1 : 200, 0.5 ml. are given diluted with the dose of streptomycin and 5–10 ml. of normal saline. When the strength of the fibrinolysin is less, proportionately greater amounts are given. With regard to frequency of administration, some cases have been given fibrinolysin intrathecally every time the streptomycin was given, while other cases have received it daily for the first fortnight of treatment only. Fibrinolysin has been given by the lumbar route, intracysternally, and into the lateral ventricles without apparent ill-effect.

Discussion

With regard to the various ways of demonstrating potency, both absolute and relative, of different yields of fibrinolysin, the following quotation from Macfarlane (1948) seems apposite: " The real or imaginary components of a theoretical mechanism can be separated to the investigator's satisfaction with little difficulty and allowed to interact in endless permutations and combinations. Each experiment suggests another ; always the intangible solution seems just within reach and the experimenter is led deeper and deeper into his own, often unjustified, interpretations of his findings." With this observation in mind, only the crudest method of assay is mentioned here, and the many experiments carried out with varying amounts of components have been omitted.

Twelve batches of 100 ml. of fibrinolysin have now been prepared, and while no two batches have been strictly comparable in strength there has been a gradual increase of potency over the series. Thus, in the first batch prepared activity was demonstrable only at a dilution of 1 : 20, batch 12 was active with the same clot at 1 : 320, while the activity of the remaining batches fell between these figures. The one toxic batch prepared was discarded with its toxicity unexplained ; it was bacteriologically sterile, and with the same operators, material, and apparatus for every batch prepared no useful line of investigation suggested itself.

It is unlikely that fibrinolysin prepared by this method does not contain varying amounts of other streptococcal toxins. Some of these may be destroyed by heat without detriment to the fibrinolysin, but as the preparation seems to have so little deleterious effect on tuberculous meninges this step has not been thought necessary.

An interesting and disappointing observation has been the infrequency with which fibrinolysin has been able to lyse the fibrin clots formed on standing in the cerebrospinal fluid from tuber-culous meningitis, while control plasma clots are lysed. Such cerebrospinal fluid has been tested without success for evidence of antifibrinolytic activity. The observation, taken with the failure to lyse plasma clots from a tuberculous guinea-pig, suggests that there may be some qualitative difference between normal fibrin and that in tuberculosis, although the meningeal exudate is fairly readily lysed.

Fibrinolysin has been used almost entirely for tuberculous meningitis, but its possibilities in other conditions with undesirable fibrin deposition have not been overlooked. In plastic peritonitis and pneumococcal empyema, for example, it might have a therapeutic place, and such cases are being investigated as they are encountered. It is possible, too, that more potent, or more specific, fibrinolysin may be obtained from organisms other than the streptococcus, but as yet only this organism has been used.

As with the treatment of tuberculous meningitis with streptomycin alone, a long period of observation will be necessary to evaluate the efficacy of fibrinolysin as an adjuvant in this condition. Several cases have been under treatment with it for varying periods of time as yet too short to warrant any other conclusion than that it may be given intrathecally without danger, and that preliminary results have been sufficiently encouraging to justify an extended trial of the preparation.

The term fibrinolysin is used here to denote the bacterial product in the presence of which lysis of a fibrin clot occurs. It is probable that this lysis depends upon a compound enzyme system and that the bacterial factor is an activator of profibrinolysin, or plasminogen (Lack, 1948).

Summary

The preparation and assay of fibrinolysin from haemolytic streptococci are described.

Investigation of some of its properties and mode of action are outlined.

The therapeutic possibilities of fibrinolysin, particularly with regard to tuberculous meningitis, are briefly indicated.

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REVIEWS

Post-mortem Appearances. By Joan M. Ross, M.D., B.S. (Lond.), M.R.C.S., L.R.C.P. Advisor in Pathology to the Ministry of Supply. Late Reader in Pathology. University of London; Morbid Anatomist to Royal Free Hospital; Assistant Pathologist to St. Mary's Hospital. Fifth Edition. 1948. London: Geoffrey Cumberlege. Oxford University Press. Pp. 308. Price 8s. 6d.

This book, which has enjoyed popularity for over twenty years, needs no introduction. The new edition, of similar external and internal appearance to its predecessors, has been revised and brought up to date by the inclusion of certain pathological conditions upon which the recent war has shed highlights, for example traumatic uraemia, starvation (shades of Belsen!) and fat-embolism, where Robb-Smith's test is conveniently described. But if it be decided (p. 121) to include the rickettsial diseases, why not some of the other infections encountered abroad?

A few suggestions and criticisms are added for the sixth edition. The weight of the thymus (p. 290) does not decrease after the second year, but after puberty. The atrophy of the suprarenals in Addison's disease (p. 193) is not " simple," as in Simmonds' Disease, but of a toxic character. In the section on the anaemias it would be helpful, by way of introduction, to indicate the normal amount and distribution of haemopoietic marrow in the femur, and its variation with age. Lastly, in the gloriously simplified gliomas, for " astroblastic " read " astrocytic "; the " spongioblastic glioma " (presumably multiforme) infiltrates, and is very rarely so circumscribed as to suggest encapsulation.

The section on still births and neonatal deaths is particularly useful.

DOROTHY S. RUSSELL.

Practical Section Cutting and Staining. By E. C. Clayden, F.I.M.L.T., Senior Technician in the Morbid Histology Department of the Bland-Sutton Institute of Pathology, The Middlesex Hospital, London. 1948. London: J. and A. Churchill, Ltd. Pp. 129. Price 9s.

This manual is designed for beginners in histological technology who are preparing for the I.M.L.T. examinations. It tells them how to master the various embedding methods, microtomes, and staining techniques that are essential in their training. The writer is obviously well versed in his craft and competent to guide the tyro round the various pitfalls of section-cutting. The artifacts (Figs. 10 and 11) known, apparently, as " scores " and " chatters " respectively are all too familiar to microscopists: it is satisfactory to find their causes listed with a view to their elimination.

While it is recognized that different tastes for various procedures may obtain in different laboratories, it is suggested (p. 43) that the picric acid method for the removal of formalin pigment from sections is preferable to the use of strong alkali which tends to bring sections off the slide. In a future edition it would be desirable to add methods for the demonstration of chromaffin tissue and Golgi apparatus; also the Marchi technique for degenerating myelin. In the staining of fatty substances the Schultz, Nile-blue sulphate, and Lorrain-Smith Dietrich methods might usefully be added.

In offering these suggestions the reviewer acknowledges the helpful comments of senior technicians in her own laboratory, who are in agreement that the work will meet a real need.

DOROTHY S. RUSSELL.

Die konstitutionellen Vergrösserungen umschriebener Körperabschnitte. By Prof. Dr. Ferdinand Adalbert Kehrer, Director of the Psychiatric and Neurological Clinic of the University of Münster (Westf.). Stuttgart: Georg Thieme Verlag, 1948. Pp. 293, with 56 figures. Price, bound, DM 28.

Under the heading "Constitutional Enlargements of Circumscribed Parts of the Body" the author has discussed the nature, characteristic features, classification, terminology, and differential diagnosis of a great host of constitutional and developmental, often hereditary, dysplastic, hyperplastic or hypertrophic or redundant abnormalities of growth. Some of the corresponding hypoplastic abnormalities, such as lipodystrophia, he has also referred to in order to elucidate the subject, but he says that he hopes to deal with these thoroughly in a second part of the book. In regard to the rarity of some of the conditions which he has considered, he points out that the scientific importance of constitutional and developmental anomalies does not depend on their frequency. In regard to explanatory theories he asks whether a writer ought to put forward more suggestions than can be well supported.

The above remarks are sufficient to point out that this book, with its wealth of references, should find a place in every general medical library. The conditions dealt with include hereditary elephantiasis, so-called trophoedema, circumscribed obesity, lipodystrophia, adiposis dolorosa, erythrocyanosis frigida crurum feminarum, acrocyanosis, clubbed fingers, hypertrophic osteoarthropathy, various local circumscribed enlargements or gigantisms, including gynaecomastia, and very many other conditions (described or referred to in discussion.)

We hope that the author's monograph will stimulate general interest in the subject, and that a second edition will appear, as well as his promised Part II. An index would be advisable, if possible.

F. PARKES WEBER.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

A Clinical Evaluation of a Recent Sulfonamide: Nu-445. RODGERS, R. S., and COLBY, F. H. (1948). *J. Urol.*, 59, 659.

The authors have used the new sulphonamide, "Nu-445" (3,4-dimethyl-5-sulphanilamido-isoxazole) in 20 cases of urinary infection caused by Gram-negative organisms, chiefly *Bacterium coli*, *Proteus vulgaris*, and *Pseudomonas pyocyanea*. By *in vitro* experiments, they found that the drug was most effective at a *p*H of between 7 and 8. The urine of all patients was therefore rendered alkaline during the treatment by the administration of sodium citrate. The usual daily dose of Nu-445 was 4 g., but some patients received 8 g. and others even 12 g. daily. There was no response in 13 cases, but 7 showed definite improvement. There appeared to be no relation between the levels of the drug in blood or urine and the clinical results. *Thomas Moore.*

Treatment of Persistent Colon Bacillus Infections of the Urinary Tract by Sulfasuxidine and Streptomycin. CROWLEY, E., and O'CONOR, V. J. (1948). *Surg. Gynec. Obstet.*, 86, 224.

The authors conclude that there is "an unusual efficacy" in the combined administration of sulphasuxidine and streptomycin in otherwise resistant bacillary infections of the urinary tract.

Administration of Chloromycetin to Normal Human Subjects. LEY, H. L., SMADEL, J. E., and CROCKER, T. T. (1948). *Proc. Soc. exp. Biol., N.Y.*, 68, 9.

Chloromycetin in the Treatment of Patients with Typhus Fever. SMADEL, J. E., LEON, A. P., LEY, H. L., and VARELA, G. (1948). *Proc. Soc. exp. Biol., N.Y.*, 68, 12.

Two normal male subjects received a prolonged course of chloromycetin beginning with a single dose of 1 g., followed by 1 g. daily (divided into 0.2 g. doses 4-hourly) for 10 days. The amounts of the drug in blood and urine were determined by a method in which the inhibition by the antibiotic of the growth of *Shigella paradysenteriae* (Sonne) is measured. Maximum levels of 5 to 10 μg. per ml. were obtained in blood within 2 hours of the initial dose, this level falling rapidly until none was detectable at 8 hours. Maximum levels in urine of about 200 μg. per ml. were obtained.

On the results obtained in a limited number of cases it is concluded that chloromycetin is relatively safe in the dosage employed; the results were sufficiently encouraging to warrant further clinical trials. *R. Wien.*

Fibrin Web Culture of Tubercle Bacilli from Exudates in So-called Idiopathic Pleurisy. (Die Fibrinspinngewebshäutchen-kultur von Tuberkelbazillen aus Exsudaten bei sog. idiopathischen Pleuritiden.) SULA, L. (1947). *Schweiz. Z. Path. Bakt.*, 10, 125.

The author describes a method of culturing tubercle bacilli from the fibrin web of pleural exudates, so that up to 300 ml. of exudate may be examined in a single culture. An ascitic fluid nutrient medium is used, and the culture sealed. Colonies of tubercle bacilli can be seen in the fibrin web after 14 to 20 days' incubation. By this method tubercle bacilli were grown from 23 out of 47 cases of so-called idiopathic pleurisy.

Primary Atypical Pneumonia. A Report of 112 Cases with a Positive Cold Agglutination Reaction. (In English.) LAURELL, G. (1948). *Acta med. scand.*, 130, 299.

The author studied 112 patients with suspected primary atypical pneumonia who showed a positive cold agglutination reaction, of a titre of at least one-eighth to one-sixteenth, during convalescence after it had been negative in the acute stage, or who showed a rising titre in convalescence. The earliest positive cold agglutination reaction was obtained from the eighth to the twenty-eighth day, the majority occurring in the second and third weeks. The highest titre occurred from the tenth to the twenty-eighth day and the first significant drop between the third and fifth weeks. The titre was not related to the maximum temperature but showed some correlation with the duration of fever and the white cell count. *J. Maclean Smith.*

Some Virus Diseases During Pregnancy and Their Effect on the Foetus. (Nagra virussjukdomar under graviditet och deras verkan på fostret.) GRÖNVALL, H., and SELANDER, P. (1948). *Nord. Med.*, 37, 409.

The authors conclude that rubella, mumps, infective hepatitis, and anterior poliomyelitis may injure the foetus, though rubella in this respect may be much less dangerous in Sweden than it seems to be in Australia; in the latter country it has been suggested that rubella early in pregnancy is an indication for inducing abortion.

[See also Landtman, *Arch. Dis. Childh.*, 1948, 23, 237, who found that in a series of 73 mothers giving birth to malformed children, 12 had had infectious diseases during the first three months of pregnancy.]

BIOCHEMISTRY

Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of Patients with Rheumatoid Arthritis. ROSE, H. M., RAGAN, C., PEARCE, E., and LIPMAN, M. O. (1948). *Proc. Soc. exp. Biol., N.Y.*, 68, 1.

An agglutination test is described which is specific for active rheumatoid arthritis. A 1% saline suspension of sheep erythrocytes is prepared from fresh defibrinated sheep blood by washing the cells 3 times in saline, centrifuging, and resuspending. A similar 1% suspension

of erythrocytes sensitized by the addition of anti-sheep rabbit serum is prepared, 2 units of haemolysin being used. With each of these 2 suspensions, 1 plain and 1 sensitized, the patient's fresh serum (inactivated by heating at 56° C. for 30 minutes) is titrated. After incubation at 37° C. for 1 hour the tubes are left in a refrigerator at 4° C. overnight; the end-point is read immediately after removal from the refrigerator by tapping the tube with the finger and estimating the degree of agglutination.

In 27 cases of active rheumatoid arthritis the D.A.T. was 16 or more; in 8 of 16 cases of inactive rheumatoid arthritis the D.A.T. was over 16; in 2 cases of active Still's disease the titre was 128, and in 1 in remission the titre was 4. The only 1 of 5 cases of ankylosing spondylitis with peripheral joint involvement was also the only one in which the D.A.T. exceeded 16. In other diseases, including rheumatic fever, infectious mononucleosis, and other forms of arthritis, titres were low. Thus the test appears to be specific and related to activity of the disease.

Plasma Proteins in the Normal Newborn Infant. (La protidemia del neonato normale.) BRUSA, P. (1948). *Med. ital., Milano*, 28, 65.

The author reports on plasma-protein determinations in 18 normal, full-term, newborn infants. The subjects of the investigation were all healthy and breast-fed.

Determination at the age of 3 to 4 days yielded the following results: total plasma protein average 6.43 g. per 100 ml. (range 5.94 to 6.94); albumin average 3.71 g. per 100 ml. (range 3.12 to 4.14); globulin average 2.78 g. per 100 ml. (range 2.22 to 3.64). A comparison with corresponding values for adults shows that total protein and albumin figures are lower while globulin figures are higher than the average adult levels. Determinations at 16 to 17 days gave the following results: total protein average 6.08 g. per 100 ml. (range 5.34 to 6.56); albumin average 3.40 g. per 100 ml. (range 3.04 to 4.00); globulin average 2.79 g. per 100 ml. (range 2.10 to 3.30).

Hypo-albuminaemia is probably and principally a manifestation of hepatic immaturity and functional incapacity to manufacture plasma proteins. The large amounts of antibodies of maternal origin present in the newborn infant and associated with the globulin fraction of the plasma proteins may be responsible for this initial hyperglobulinaemia.

[C. Smith, *The Physiology of the Newborn Infant*, Springfield, 1946, pp. 179–200, and 286, states that the hypoproteinaemia of the newborn infant is essentially the result of globulin deficiency, and gives various references.]
P. E. Polani.

The Guterman Test for Pregnancy. MERIVALE, W. H. H. (1948). *Brit. med. J.*, 1, 685.

The Guterman Test in Threatened Abortion. A Report Based on 100 Consecutive Cases. BENDER, S. (1948). *Brit. med. J.*, 1, 683.

Both authors conclude that this test is unreliable as a routine test for the diagnosis of pregnancy. In 33 patients with threatened abortion the first author obtained a positive test in 20, and of these 17 continued their pregnancy, whilst of 13 with negative results 11 aborted. The second author claims that if cases of threatened abortion are treated with progesterone only if the excretion of the hormone is low, abortion will occur only in 18 per cent. If no such division is made 41 per cent will abort if no progesterone is used and 45 per cent if it is used indiscriminately in all cases.

The Renal Excretion of Potassium. MUDGE, G. H. FOULKS, J., and GILMAN, A. (1948). *Proc. Soc. exp. Biol., N.Y.*, 67, 545.

With a urea diuresis, potassium clearances were 5 to 10% above creatinine clearances and, at a diuresis with minute volumes of 18 to 35 ml., clearances were as much as 80 to 90% above creatinine clearances. The effect of alkalosis was studied in dogs receiving potassium chloride by intravenous infusion; in these animals potassium clearances were greater than the filtration rates. It is concluded that tubular secretion of potassium does occur.
G. Loewi.

HAEMATOLOGY

Histochemical Methods Applied to Hematology. RHEINGOLD, J. J., and WISLOCKI, G. B. (1948). *Blood*, 3, 641.

This paper is a good review of how histochemical methods are being applied to blood cells. The specific reactions with Sudan black B, Feulgen's reagent and ribonuclease, and Gomori's procedure for phosphatases and the Bauer-Feulgen test for glycogen are considered.

Studies on the Megakaryocyte. I. The Normal Granulopoiesis of the Megakaryocyte. SCHWARZ, E. (1948). *Arch. Path.*, 45, 333.

The development of granules in megakaryocytes is a sign of a cell's increasing maturity, as it is with the polymorph series. The staining reaction of the granules is not that of chromatin; and the nucleus plays no part in their formation.

In Vitro **Study of Bone Marrow.** III. Erythropoiesis *in vitro* of Sternal Marrow from Cases of Pernicious Anemia and Lymphatic Leukosis Under Therapy. CLEMMESEN, J., ESPERSEN, T., and PLUM, C. M. (1948). *Blood*, 3, 155.

Using Locke's fluid with the addition of 1% serum the authors have studied the development of non-nucleated erythrocytes in their marrow cultures. They found that normal marrow produced less erythrocytes when serum from a case of pernicious anaemia was used in the culture medium than when normal serum was used, and that the pernicious anaemia marrow was more active in normal serum than in autogenous serum. After treatment with liver extract there was a return to normal. Marrow and serum from two patients with lymphatic leukaemia behaved in the same way. The leukaemic marrow was more active in normal serum, and the serum retarded the development of erythrocytes in normal marrow.

Temporary Remissions in Acute Leukemia in Children Produced by Folic Acid Antagonist, 4-Amino-pteroylglutamic Acid (Aminopterin). FARBER, S., DIAMOND, L. K., MERCER, R. D., SYLVESTER, R. F., and WOLFF, J. A. (1948). *New Engl. J. Med.*, 238, 787.

Ten out of 16 children improved both clinically and haematologically when aminopterin was administered, and details of five who responded well are given. One patient remained well for as long as 43 days, but the remissions are not expected to be more than temporary. The drug is a dangerous one and has a marked depressive effect on all the cells in the bone marrow, and great caution is necessary in its administration. Severe stomatitis is a common toxic manifestation.

Preliminary Results of Treatment of Acute Leucoses by Exsanguination-transfusions. (Premiers résultats du traitement des leucoses aiguës par les exsanguino-transfusions.) CROIZAT, P., REVOL, L., DURAND, J., CHABANON, R., and MOREL, —. (1948). *Lyon méd.*, 180, 477.

A report is given of 5 patients with acute leukaemia treated by exsanguination transfusion. In each case the blood and bone marrow picture became almost normal. The longest remission lasted for about 3 months.

Rapid Test for the Demonstration of Sickle Cells and its Clinical Significance. SINGER, K., and ROBIN, S. (1948). *J. Amer. med. Ass.*, 136, 1021.

A suspension of *B. subtilis* is mixed on a slide with the blood to be tested and the sealed preparation incubated at 37° C. Reduction in oxygen tension by the respiration of the bacteria rapidly causes sickling in the presence of the sickle-cell trait.

Studies in Iron Transportation and Metabolism. VI. Absorption of Radioactive Iron in Patients with Fever and with Anemias of Varied Etiology. DUBACH, R., CALLENDER, S. T. E., and MOORE, C. V. (1948). *Blood*, 3, 526.

MORBID ANATOMY AND HISTOLOGY

Necrosis of the Brain due to Radiation Therapy. PENNYBACKER, J., and RUSSELL, D. S. (1948). *J. Neurol. Neurosurg. Psychiat.*, 11, 183.

Five cases of necrosis of the brain following radiation therapy are reported. They were treated in different centres with accepted techniques and dosages. In each case there was a long latent period between the radiation treatment and the onset of clinical signs of necrosis. The shortest interval was 9 months, the longest 5 years. The pathology of the necrosis appears to be related to reactions in the smaller blood vessels, in which collagenous thickening, fibrinoid necrosis, and thrombosis are conspicuous. It is suggested that radiation should be reserved for inoperable tumours and those cases in which no further surgical procedures are contemplated.

Myoepithelial Sweat Gland Tumor: Myoepithelioma. Report of Three Cases with a Review of the Literature. LEVER, W. F. (1948). *Arch. Derm. Syph., Chicago*, 57, 332.

It is suggested that the group of benign sweat-gland tumours in which the myoepithelial cells represent the majority of the cellular elements should be called "myoepitheliomata." The paper deals with the occurrence of myoepithelial cells in many types of sweat-gland tumour, with reports of 12 cases of myoepitheliomata abstracted from the literature of the subject, and with details of 3 observed cases. There is a discussion on the classification of apocrine gland tumours.

"Basaliomata" of Spiegler. (Basaliome vom. Typus "Spiegler.") GREITHER, A. (1948). *Arch. Derm. Syph., Wien*, 187, 224.

The various synonyms are discussed (cylindroma, basal-cell epithelioma, multiple benign epithelioma, trichobasalioma), and the term basalioma (Spiegler) is suggested as being more correct pathologically. Histologically, the structure of the tumours resembled that of hair follicles, but there was not enough evidence to show that the growths originated from the latter. A feature in one case was the presence of the so-called mixed parotid tumour, but no aetiological connexion was thought likely. None of the cases reviewed by the author, including his own 2 cases, showed definite malignant changes. *G. W. Csonka.*

Myxoma, the Tumor of Primitive Mesenchyme. STOUT, A. P. (1948). *Ann. Surg.*, 127, 706.

The author defines a myxoma as a true neoplasm composed of stellate cells set in a loose mucoid stroma through which course very delicate reticulin fibres. Denser areas with larger numbers of spindle cells and less mucoid material may be encountered in a myxoma, but the presence of recognizable differentiated cells, such as chondroblasts, lipoblasts, or rhabdomyoblasts, rules out the diagnosis. Myxomata do not metastasize, but may kill by pressure or direct spread into vital organs. Using these criteria, the author found 49 myxomata in the records of the surgical pathological laboratory of Columbia University. Over 100 cases of myxomata involving the heart, and another 95 excluding the heart, have been found in the literature. The common sites for myxomata are the heart, the subcutaneous tissues, the bones (especially the jaws), the urogenital tract (usually the bladder), and the skin. The sex incidence is about equal, the age incidence from infancy to senility, and the size very variable. The average duration of the tumour has been 4 years, the actual duration ranging from 2 weeks to 37 years. A pre-operative biopsy is advocated.

The paper includes six well-illustrated case reports.

R. S. Handley.

Allergic Hyperglobulinosis and Hyalinosis (Paramyloidosis) in the Reticulo-endothelial System in Boeck's Sarcoid and Other Conditions. A Morphologic Immunity Reaction. TEILUM, G. (1948). *Amer. J. Path.*, 24, 389.

Hyperglobulinemia, Periarterial Fibrosis of the Spleen, and the Wire Loop Lesion in Disseminated Lupus Erythematosus in Relation to Allergic Pathogenesis. TEILUM, G. (1948). *Amer. J. Path.*, 24, 409.

Periarterial fibrosis of the spleen and lymph nodes, such as was first described by Libman and Sacks in disseminated lupus erythematosus, occurs also in Boeck's sarcoid. It is associated with the increase in γ globulin in the blood which exists in both diseases. The lesion is due to the accumulation of hyalin (in concentric rings), and results from the precipitation of the abnormal globulin. Hyalin (or pre-hyalin) is also found in relation to the granulomatous foci, and in the early stages of its formation may be mistaken for caseous material. The abnormal globulin in states of hyperglobulinosis is formed by plasma and other cells of the reticulo-endothelial system, and these cells were often increased in the vicinity of the hyalin.

In the second paper the author maintains that the diffuse lesions (such as the periarterial fibrosis of the spleen, and wire-loop lesions of the glomeruli) are not the result of widespread degeneration of collagen but are due to an infiltration with hyalin. He believes that the hyalin is formed by precipitation of globulin, which is present in excess. He regards the hyperglobulinosis as indicating a state of hyperimmunity, and as "allergic," in the wider use of the term. *D. M. Pryce.*

CORRECTIONS

The following errors occurred in the article by J. V. Dacie and J. C. White on " Erythropoiesis with Particular Reference to its Study by Biopsy of Human Bone Marrow" in the February, 1949, issue, Vol. 2.

On Plate IV the legend to Fig. 3 has been omitted, and should read:

Fig. 3 shows primitive cells and maturing cells, some of them megaloblasts; also cells with pyknotic nuclei and degenerating cytoplasm.

On page 27, second column, all the references to Plate III should have been to Plate IV.

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saccharide-containing substance which is either mucoprotein or glycoprotein in the cytoplasm of plasma cells and reticulum cells, in Russell bodies, and in the Kurloff bodies of guinea-pig lymphocytes. The work is based on the observation that the cytoplasm of a proportion of plasma cells stains pink by the periodic-acid-Schiff technique of McManus (1946, 1948) and Hotchkiss (1948), which demonstrates polysaccharides in the tissues. From the cytoplasm of plasma cells to Russell bodies in plasma cells is a logical step, while Kurloff bodies and Auer bodies in human myeloblasts were examined on account of a suggestion by Downey (1938) that all three are possible homologues.

Russell-Bodies

The development of intracellular acidophil hyaline bodies, now known to take place in plasma cells, has been recognized since 1890 when William Russell, then pathologist to the Edinburgh Royal Infirmary, first described them as the " characteristic organisms of cancer." He regarded them as fungi, and for their demonstration used eosin and logwood or carbol fuchsin and iodine green. Russell examined a large variety of tissues and came to the conclusion that the acidophil bodies were not found in degenerations or in typhoid, tubercle, or inflammatory processes in general. Exceptions to this rule he found in gummata and in a case of " gelatinous degeneration of the knee joint." Simple tumours did not contain the bodies; but he found numerous examples in a wide variety of malignant growths. He noted their distribution especially in the small round-cell infiltration in the margin of cancers

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: says, in little clusters of two, up to twenty or more, and the larger clumps are held together by a delicate cementing substance which stains faintly."

Since then many views have been expressed as to the precise nature of Russell bodies and three main theories have been evolved. First, Downey (1911), Kingsley (1924), and others regard the bodies as pathological secretions or as aggregations of the normal secretions formed as the result of degeneration in the parent plasma cell. Subsequently, it is thought, death of the parent cell liberates the bodies into the tissues. Secondly, Jordan and Speidel (1929) and Dawson and Masur (1929) regard the cells which contain Russell bodies as haemocytoblasts which have failed to transform in the normal manner into erythrocytes or granulocytes. Thirdly, Michels (1935) considers that the bodies are red blood corpuscles which have been taken up by phagocytic plasma cells.

A review of the literature shows that there is some tendency to refer to Russell cells instead of Russell bodies. It is suggested that the latter is the better term, for two reasons. First, Russell himself described bodies and not cells, and, secondly, the parent is now known to be the plasma cell, and whatever the true origin of these there is no need to disinherit them on account of their cytoplasmic inclusions.

Sources of Russell Bodies.—Plasma cells containing Russell bodies are found constantly in a wide variety of chronic infections and in the granulomata (Fig. 3). They are to be found in small numbers in the normal intestinal submucosa of man and in larger numbers in the membrana propria of the abomasum, the fourth or true stomach, of ruminants. In this site they are the

Schollenleukocyten of Weill, described in the abomasum of the sheep. They occur constantly, as described by Russell, in the small round-cell infiltrations at the periphery of malignant tumours (Fig. 1). Russell bodies occur also in the abnormal plasma cells of plasmacytoma, where, according to Willis (1948), abnormal proteins produced in the tumour cells appear as rounded fuchsinophil masses or as crystals.

The main sources of Russell bodies in these investigations have been (1) the periphery of malignant tumours, (2) various chronic inflammatory granulomata, (3) six cases of plasmacytoma.

Kurloff Bodies

Kurloff bodies were first described by Kurloff in 1889, and independently in the same year by Foa and Carbone, as inclusion vacuoles in the cytoplasm of haemic and splenic lymphocytes in the guinea-pig. They occur in from 2 to 20 per cent of the haemic lymphocytes. Bab is quoted by Ledingham (1906) as having found in the bone marrow and spleen of guinea-pigs certain vacuolated cells containing masses of ill-defined granules staining metachromatically with thionin. These Ledingham, but not Bab, regarded as Kurloff cells. Ledingham himself (1940) uses the term " cell," but I suggest, for reasons similar to those adduced for Russell bodies, that the term Kurloff body and not cell is more appropriate.

In air-dried films of guinea-pig blood, fixed in methyl alcohol and stained by Romanowsky methods, the appearance of Kurloff bodies varies considerably. In films wet-fixed in Susa, however, the body appears as a uniformly spherical isotropic globule whose diameter may equal and often exceeds that of the nucleus (Fig. 7). Only where the cells have been crushed by the process of making the film does the material contained in the bodies appear granular. I consider that the appearances described by Ledingham (1940) based on air-dried methyl alcohol fixed films stained by Giemsa are largely artifact.

There are four theories as to the nature of Kurloff bodies.

Secretory Theory.—Kurloff himself originally suggested that they were some kind of secretion (*Sekretstoff*), and it has also been considered possible that they may represent aggregations of the azurophil granules normally present in lymphocytes.

Parasitic Theory.—According to Ledingham, Mochkovski in 1937 suggested that the bodies were rickettsial and proposed the name *Ehrlichia*

kurlovi in honour of the discoverer, who worked in Ehrlich's laboratory.

Phagocytic Theory.—The bodies have been considered to represent phagocytosed red cells or nuclear remnants derived from other cells (Lazzeroni, 1935).

Nuclear Theory.—This theory derives from Leinàti (1932), who regards the body as representing half the nucleus of the lymphocyte which has undergone pycnosis. Lymphocytes with double nuclei connected by a thin band of chromatin are not uncommon in the guinea-pig.

In this investigation, the sole source of Kurloff bodies has been films of adult guinea-pig's buffy coat, wet-fixed in Heidenhain's Susa fixative or in saturated aqueous basic lead acetate.

Method

The use of the periodic acid Schiff reagent (P.A.S.) method in histology was first described by McManus (1946) for the demonstration of mucin and by Hotchkiss (1948) for a wide variety of polysaccharide structures.

By this method the monosaccharide components of polysaccharide structures remaining in the tissues after the use of ordinary aqueous fixatives are oxidized by periodic acid (HIO_4) to polyaldehydes, and the latter are combined *in situ* with Schiff's reagent (leucofuchsin) to form a red substituted dye. If the periodic acid method is used as described in the appendix on paraffin sections after aqueous fixatives, then five groups of substances may be expected to give positive results and these will not be visible in control sections unexposed to periodic acid. The five groups are given below.

I. Polysaccharides.—Of this group glycogen is the only member remaining after aqueous fixation and paraffin embedding.

II. Mucopolysaccharides (simple and complex).—These are defined by Meyer (1938) as polysaccharides containing hexosamine as one component, occurring either free (simple) or in combination with protein substances of higher molecular weight and as esters of sulphuric acid (complex). Both simple and complex acid mucopolysaccharides contain uronic, usually glycuronic, acid as their second carbohydrate component. They include hyaluronic acid, intestinal mucins, chondroitin sulphuric acid, and heparin. The smaller fraction of gastric mucus is included here as a neutral mucopolysaccharide.

III. Mucoproteins (Mucoids).—These are defined by Meyer as substances containing mucopolysac-

charide in firm chemical union with a peptide where the hexosamine content is greater than 4 per cent. The mucoproteins contain hexosamine and hexose as their carbohydrate component as a rule, though uronic-acid-containing mucoids do occur. In this group are included the pituitary and chorionic gonadotropic hormones, submaxillary gland mucoids, and the serum mucoproteins seromucoid and seroglycoid. The larger fraction of gastric mucus is a mucoprotein.

IV. Glycoproteins.—These are divided from the mucoproteins on the basis of their hexosamine content of less than 4 per cent. They include fractions of serum albumin and serum globulin. According to Meyer (1945) simple proteins containing hexoses without hexosamine have not been found in nature.

V. Glycolipids.—These substances, on hydrolysis, yield one molecule each of fatty acid, sphingosine, and galactose or glucose. They stain positively with the periodic acid method by virtue of the sugar. The cerebrosides, which are the main members of the group, occur in a variety of tissues outside as well as within the nervous system. After fixation, even in formalin alone, their solubility in fat solvents is altered so that they may still be present in paraffin sections. Hotchkiss suggests that the alcoholic solutions used in his periodic acid method complete the removal of those traces of cerebroside and other polysaccharide-containing-lipoids which may remain in paraffin sections. Unfortunately, I have found evidence, at least in the case of the anterior hypophysis, that this is untrue.

The three substances hexuronic acid, hexosamine, and hexose are responsible for the development of colour when the periodic acid method is employed. *In vitro,* equimolecular portions of hexose and hexosamine give the same intensity of colour when oxidized by periodic acid and allowed to react with leucofuchsin.

Cytochemical Distinctions.—*Group 1.*—Glycogen can be removed from sections by hydrolysis with diastase or ptyalin. During this work control sections, where necessary, have been exposed to the action of saliva for ten minutes at room temperature before applying the periodic acid or other techniques.

Group 2.—The only simple mucopolysaccharide with which we are concerned is hyaluronic acid. Experiments suggest that the amount of this substance present can be reduced by exposing sections to the action of hyaluronidase. Complex muco-

polysaccharides contain higher esters of sulphuric acid or phosphoric acid groups and are distinguished by their property of staining metachromatically in dilute aqueous solutions of thionin or toluidine blue. Lison (1936) regards metachromasia as a specific histochemical indication of the presence of higher esters of sulphuric acid, but it is apparent that the phosphoryl groups of ribonucleic acid, in some circumstances, induce metachromatic staining with dilute aqueous thionin. Hyaluronic acid is metachromatic where it occurs in the form of its sulphuric acid ester as, for instance, in the cornea. In paraffin sections, therefore, metachromasia may signify sulphuric or phosphoric acid groupings.

Further assistance may be obtained by a study of the dye-binding capacity for methylene blue measured at various pH levels (Dempsey and Singer, 1946). These authors found that mast cell granules and the ground substance of cartilage still bound methylene blue at pH 2 whereas the binding capacity of tracheal mucus was extinguished at pH 3 and that of ribonucleic acid at pH 4. The ribonucleic acid they studied was that of thyroid colloid, but I have found that the ribonucleic acid of plasma cell cytoplasm still binds methylene blue at pH 2.6. After ribonuclease, plasma cell cytoplasm is only faintly blue at pH 6. If the nucleic acids can be excluded the capacity to bind methylene blue below pH 4 almost certainly indicates sulphate ions and thus mucopolysaccharides.

Groups 3 and 4.—These two groups have been subdivided according to Meyer's arbitrary though convenient classification, and no cytochemical distinction is possible between them. Theoretically, substances in Group 3 might be expected to give more colour than those in Group 4 if their hexose components are equal. Factors such as concentration and availability apparently modify the development of colour to such an extent, however, that no conclusion can be derived from its intensity. The most important point is that substances in Groups 3 and 4 do not show metachromasia with thionin and will not bind methylene blue below pH 6.

Group 5.—The possibility that periodic acid material in paraffin sections may still belong to this group must always be recognized. Control frozen sections stained for lipid by Sudan IV or Sudan black B and compared with paraffin and frozen periodic acid sections will usually allow the lipoidal nature of the substance to be established or excluded.

Results

The results of the present series of experiments are shown in the Table. In the accounts of these findings and in the description which follows, substances in Groups 3 and 4 are referred to, jointly, as mucoprotein.

Russell Bodies.—If a tissue is chosen in which plasma cells containing Russell bodies are present in addition to the ordinary types, then intermediate states can be found between the plasma cell with faintly periodic-acid-positive cytoplasm and one containing the fully developed Russell bodies. These states are best seen in sections stained by periodic acid after ribonuclease (Figs. 2 and 4).

The stages of development appear to be as follows. The faintly positive plasma cell, which is usually normal in size, first enlarges and becomes more strongly positive. The contained material then becomes finely granular with a decrease in the total intensity of staining. The granules enlarge, increasing until they become globules the size of adult red cells or larger, and the intensity of staining rises simultaneously. Finally, the cell

TABLE
RESULTS OF THE EXPERIMENTS

Structure	Eosino-philia	Basi-philia	P.A.S.*	P.A.S. after ribo-nuclease	Pyronin	Pyronin after ribo-nuclease	P.A.S. after hyal-uronidase	P.A.S. after diastase	Meta-chrom-asia and M.B.E.†	Constitution
Plasma cell cytoplasm	+ Degree varies	+	+ Degree varies	+ Degree varies	+	Basiphilia reduced	+ No reduction	+	— About pH 6.0‡	(1) Ribonucleic acid (2) mucoprotein
Russell bodies (various sources)	+	+ Around globules	+	+	+ Around globules	Surround-ing sub-stance removed	+ No reduction	+	— About pH 6.0‡	(1) Mucoprotein surrounded by (2) ribonucleic acid
Kurloff bodies	+	—	+	+	+ Around globules	Surround-ing sub-stance removed	+ No reduction	+	— pH 6.9	(1) Mucoprotein surrounded by (2) ribonucleic acid
Tissue mast cell granules	+	Slight (H & E)	+	+	+	+	+ No reduction	+	+ Nil at pH 2.6	(1) mucopoly-saccharide
Pituitary basiphil cells	+	+	+	+	+	Basiphilia removed	+ No reduction	+	— pH 4.9‡	(1) Ribonucleic acid (2) mucoprotein
Auer bodies	Azuro-phil	—	+—							Inconclusive
Azurophil granules (lympho-cytes)	Azuro-phil	—	—							Not mucopro-tein
Blood and tissue eosinophil polym'phs	+	—	—						—	Not mucopro-tein
Paneth cell granules	+	—	— To faint +						—	Not mucopro-tein
Red blood cells	+	Reticu-locyte forms +	—	—	—	Reticulo-cytes not basiphil			—	Not mucopro-tein

* P.A.S. = Periodic acid Schiff. † Methylene blue extinction. ‡ After ribonuclease.

may be so full of globules and their colour so deep that they appear to coalesce. In some cases the process appears to stop at the stage of a cell scarcely larger than the normal plasma cell, with bright red non-granular cytoplasm. These cells appear in the majority of chronic infective conditions, and with haematoxylin and eosin staining look like eosinophils with unlobed nuclei. Similar cells containing fine eosinophil granules which are P.A.S.-positive and metachromatic with thionin occur in various small round-cell infiltrations. These are the plasma-mast cells of Krompecher described by Michels and Globus (1929) in cerebral syphilis.

Comparison with sections stained by the Unna-Pappenheim method shows that the amount of pyronin-positive material in the plasma cell (ribonucleic acid) decreases as the amount of P.A.S.-positive substance (mucoprotein) rises. Furthermore each Russell body is surrounded by, to use Russell's own words, " a delicate cementing substance which stains faintly." It is almost certain that this is the basiphilic and pyronin-positive material which is removable by hydrolysis with ribonuclease and is, most probably, ribonucleic acid itself (Fig. 5).

The substance of which the bodies are composed is unaffected by ribonuclease, diastase, or hyaluronidase, fails to bind methylene blue at pH 6, and is devoid of metachromatic properties. It is, therefore, mucoprotein or at least contains mucoprotein as a major constituent.

Kurloff Bodies.—Guinea-pig lymphocytes have basiphilic cytoplasm which is pyronin-positive ; as in the case of human lymphocytes this is due to ribonucleic acid and is removable by ribonuclease. A thin layer of this pyronin-positive ribonucleic acid surrounds each individual Kurloff body (Fig. 7a), and it is this layer which, irreversibly precipitated as a network either by the vital stains or by air drying of blood films and subsequently fixed and counterstained, has been described by various authors as the inclusion body of the so-called Kurloff cell. In fact the body within this reticulum is ribonuclease-fast and brilliantly P.A.S.-positive (Figs. 7b and 7c). It is fast also to diastase and hyaluronidase and does not show metachromasia. In preparations wet-fixed in Susa, however, and mounted in a watery medium the cytoplasm of the lymphocytes and the thin layer covering the Kurloff body show reddish metachromasia with thionin. This finding probably explains the results obtained by Bab, mentioned above.

It is concluded that the Kurloff body consists wholly or partly of mucoprotein.

Other Structures in the Table.—The remaining structures in the Table have been included for various reasons. Tissue mast cells conveniently provide themselves as controls for metachromasia in many sections containing plasma cells and Russell bodies (Fig. 6). It has been suggested by Holmgren and Wilander (1937) that mast cell granules contain the acid mucopolysaccharide heparin, and the results listed in the Table are in agreement with this view.

The pituitary basiphil cells present a problem similar to that posed by plasma cells. It must be emphasized that their granules are primarily only weakly basiphil and that their cytoplasm is secondarily basiphil by virtue of its ribonucleic acid content. As Desclin (1940) has shown, and as Dempsey and Wislocki (1945) have confirmed, this secondary basiphilia is removable by hydrolysis with ribonuclease. After such hydrolysis with ribonuclease, the true β granules, which now contain no ribonucleic acid, remain in the basiphils and are weakly stained by 5×10^{-4} M methylene blue at pH 6.4 whereas the α granules are stained strongly blue at this pH. The α granules are thus more basiphil than the true β granules at this pH. The basiphil (β) granules are strongly P.A.S.-positive both before and after ribonuclease and they resist the action of diastase and hyaluronidase. Although Bienwald (1939) was able to demonstrate in thionin-stained formol-fixed frozen sections the presence of a metachromatic substance in the basiphils, the β granules have not been found to exhibit metachromasia in either frozen or paraffin sections. The substance demonstrated by Bienwald was probably fatty material (*Fettstoffe*), as he suggested.

Pearse (1948) considers that the true β granules represent the gonadotropic hormone (F.S.H. or F.S.H.+L.H.) which has been shown by Evans and co-workers (1939), and by various other workers, to be a mucoprotein.

The results obtained with Auer bodies have been unsatisfactory for the following reasons. Cells containing Auer bodies are seldom numerous in either blood or bone marrow, and the bodies cannot be demonstrated except in air-dried films fixed in methyl alcohol and stained by one or other of the Romanowsky methods. Auer bodies in myeloblasts, located by this method, marked, decolorized, and subsequently treated with periodic acid, have been found to show a faint red colour demonstrable only by the use of green filters but not shown by the decolorized control. The azurophil granules of lymphocytes are periodic-acid-negative.

Discussion

In the light of the present work, only one of the three theories of origin of Russell bodies can be maintained. This is the secretory theory of Downey (1911) and Kingsley (1924).

Although it is clear that Russell bodies are formed in large numbers in response to abnormal stimuli, such as may be supposed to occur in the vicinity of malignant tumours or in long-continued chronic inflammation, their presence is clearly not invariably associated with obvious pathological processes. If one of the functions of the plasma cell is to produce mucoproteins, then the formation of Russell bodies may represent a disorder due to over-stimulation and over-production. The evidence presented in this paper suggests that plasma cells normally can produce mucoproteins and that the quantity is usually insufficient or too rapidly secreted for granular and globular retention stages to occur.

Most supporters of the secretory theory believe that the formation of Russell bodies is a degenerative phenomenon, and the cytological appearances certainly support such a view. The large cartwheel nucleus of the plasma cell becomes distorted by the pressure of accumulated secretion, shrinks, and becomes pycnotic in the majority of cases, so that death of the cell and liberation of its content appear to be the usual end-result.

In his work on the genesis of red blood corpuscles from eosinophil leucocytes Duran-Jorda (1943a and b, 1948) describes one of the stages of erythropoiesis as a cell, originally called by him the " stem cell " and the " plasma cell with eosinophilic granules." The situation and appearance of this cell parallel so exactly that of the plasma-cell-containing Russell bodies as to make it almost certain that they are identical. Since Russell bodies are of mucoprotein nature and since red blood cells and the granules of tissue and blood eosinophils are uniformly P.A.S.-negative, I consider that he is mistaken in including the Russell-body-containing plasma cell in his erythropoietic series.

As far as Kurloff bodies are concerned, the conclusion arrived at in these studies is that they are produced in guinea-pig lymphocytes by a process of secretion similar to that involved in the production of Russell bodies by human plasma cells. Freed from the surrounding ribonucleic acid reticulum, which has misled so many investigators, the structure of Kurloff bodies is seen to be very simple indeed. Of the four theories of origin presented, only Kurloff's original one, which regards them as a secretion, fits the evidence.

Ledingham (1940) found that the number of circulating lymphocytes containing Kurloff bodies was normally greater in female than in male guinea-pigs, and greater still in pregnant females, and that the number could be greatly increased by injection of oestrogenic substances (oestradiol). Here is evidence of a controlled stimulus to secretion of mucoprotein affecting guinea-pig lymphocytes.

The present experiments show that, apart from the Russell bodies, the cytoplasm of a proportion of normal plasma cells contains mucoprotein. This substance may have been phagocytosed by the cells or synthesized, or its precursors may have been absorbed and subsequently converted into mucoprotein. It is important to know which of these hypotheses is the true one, because if the mucoprotein is synthesized the fact may have some bearing on the possible production of antibody by plasma cells and on the possible production of abnormal globulins by plasmacytoma.

Rohr (1936) states that in the bone marrow plasma cells are specialized reticulum cells which phagocytose fat, erythrocytes, and nuclear debris; and Waldenström (1944) goes further and states that in plasmacytoma they take up and store abnormal globulins formed elsewhere in the body. The majority of workers, however, hold the opposite view. Bing and Plum (1937) noted that hyperglobulinaemia and plasma-cell increase tended to be associated, and considered that plasma cells produced the globulins. Björneboe and Gormsen (1943) demonstrated in the rabbit immunized against pneumococci that the formation of plasma cells in various tissues accompanied the rise of antibody titre. They were unable to increase the number of plasma cells by injection of globulins, and concluded that antibody globulins are formed by plasma cells but that storage does not take place. Ranström (1946) noted the discrepancy between the views of Waldenström and the observations of Björneboe and Gormsen, and agreed with the latter, though it is fair to note that the findings of their second experiment can only be applied to the particular globulins employed and not to globulins in general. Teilum (1948) also holds that plasma cells and other reticulo-endothelial cells produce the various antibodies and also hyaline and amyloid.

It is evident that the weight of opinion upon plasma cells is against storage and in favour of secretion. It is considered that the inverse relationship between ribonucleic acid and mucoprotein, noted especially in plasma cells which are forming Russell bodies, supports synthesis and not

absorption. In order to explore the possibility that connective tissue mucin (hyaluronic acid) might be absorbed to form Russell bodies in connective tissue plasma cells, the action of hyaluronidase upon these was tested and found to be absent. This evidence is against the absorption of hyaluronic acid and in this respect favours secretion.

In cases of plasmacytoma the abnormal circulating globulins may belong to the α, β, or γ fractions, and Blix and others (1941) have shown that normal β and γ fractions contain carbohydrate. Normal globulins may therefore be glycoglobulins. If the abnormal globulins of plasmacytoma contain sufficient carbohydrate they should be P.A.S.-positive. In the present work I have examined six cases of plasmacytoma and found a positive reaction in a small proportion of the plasma cells in four of them. In the fifth and sixth cases, the only ones in which undecalcified tissues were examined, a large proportion of the plasma cells and reticulum cells showed a positive reaction (Figs. 8a and 8b). It seems probable that the acid used for decalcification hydrolyses the mucoprotein and renders the P.A.S. reaction negative. It is hoped that a study of further cases will confirm these findings.

Summary

1. The periodic acid Schiff method of McManus and Hotchkiss has been used together with other techniques to investigate the cytochemistry of plasma cells, Russell bodies, and Kurloff bodies. A number of other structures whose chemistry throws light on the problem have been investigated simultaneously.

2. Evidence is presented that Russell bodies in human plasma cells and Kurloff bodies in guinea-pig lymphocytes consist of mucoprotein probably secreted by the parent cell. The implication of these findings on the various theories of origin of the two types of body are briefly discussed.

3. The presence of mucoproteins in the cytoplasm of human plasma cells is demonstrated, and it is suggested that these mucoproteins are polysaccharide-containing globulins (glycoglobulins, mucoglobulins). The question whether the mucoglobulin is secreted or absorbed by plasma cells is considered and evidence adduced in favour of the former.

Appendix

Periodic Acid Schiff (McManus, Hotchkiss, modified).—For paraffin sections mercurial fixatives are used for choice, but formalin is quite adequate. For blood films wet fixation in Susa for 10 minutes (White, 1947) is employed. The films are preferably stained at once.

After removal of mercury salts by means of iodine and thiosulphate, (1) bring to 70 per cent alcohol; (2) leave for 5 minutes in periodic acid solution (A) at room temperature; (3) flood with 70 per cent alcohol and transfer to reducing rinse (B) for 1 minute; (4) flood with 70 per cent alcohol and leave for 15 to 45 minutes in fuchsin-sulphite solution (C); (5) wash in running water for 10 to 30 minutes; (6) stain the nuclei in 0.5 per cent celestin blue in 5 per cent aqueous iron alum (Lendrum and McFarlane, 1940) for 1 minute, followed by Meyer's haemalum for 1 minute. The object of the short staining period is to avoid emphasis of cytoplasmic basiphilia to a degree sufficient to obscure the red colour of Schiff-positive substances. The times in stage 6 may be increased without limits after ribonuclease. (7) Differentiate strongly in 2 per cent acid alcohol, wash, dehydrate, clear in xylol, and mount in D.P.X.

(A) HIO₄ ... ... 400 mg.
Aq. dest. ... ... 10 ml.
M/5 sodium acetate buffer ... 5 ml.
Pure ethyl alcohol ... ... 35 ml.
(Keeps in the dark at 4° C. for at least 14 days.)

(B) KI ... ... 1 g.
Na₂S₂O₃.5H₂O ... ... 1 g.
Aq. dest. ... ... 20 ml.
Pure ethyl alcohol ... ... 30 ml.
2N HCl ... ... 0.5 ml.
(The precipitate of sulphur which forms is ignored. This solution must be reacidified if necessary from time to time.)

(C) The Schiffs solution of Feulgen (see Lison, 1936, p. 178) or any of its modifications may be employed.

Ribonuclease.—This was used as a 0.5 per cent solution in isotonic veronal acetate buffer at *p*H 6.75. To destroy any remaining proteolytic action the enzyme is heated for 3 minutes at 100° C. in aqueous solution, and this is added to double-strength acetate buffer to make the solution for use (White, 1947). A crystal of thymol is added to the solution in a coplin jar to prevent bacterial contamination.

Sections are incubated at 37° C. for 1 hour.

Hyaluronidase.—500 mg. of dried bull's testis is added to 50 ml. of isotonic veronal acetate buffer at *p*H 6.99 and stirred gently with a glass rod. The mixture is left for 30 minutes for undissolved solid matter to settle; it is then decanted, and sufficient proflavine (2.8 diamino-acridine hydrochloride) is added to give a faint yellow tinge. Hyaluronidase is quickly destroyed by shaking, but its action is unimpaired by bacteriostatics of the acridine series.

Sections are incubated at 37° C. for 24 hours. A 30 to 40 per cent reduction of P.A.S.-positive colour is obtained in frozen sections of umbilical cord and cornea, and this reduction is not shown in control sections incubated for 24 hours in buffer at *p*H 6.99.

Unna-Pappenheim.—Sections, preferably fixed in Helly or other mercurial fixatives, are stained for 30 minutes in the following solution:

Methyl green (C.I. No. 684)	...	0.15 g.
Pyronin G. (C.I. No. 739)	...	0.25 g.
·90% ethyl alcohol	... ...	2.5 ml.
Glycerine	... ...	20.0 ml.
0.5% aqueous phenol to 100 ml.		

The solution is boiled for 2 minutes, and filtered before use.

Metachromasia.—Sections fixed in mercurial fixatives are stained for 1 hour in 0.25 per cent aqueous thionin (C.I. No. 920), or in 0.5 per cent aqueous toluidine blue (C.I. No. 925), and are mounted in water for examination or in glycerine jelly for permanency.

For thionin staining, films of guinea-pig's buffy coat were fixed in saturated aqueous basic lead acetate, which is said to facilitate the demonstration of metachromasia.

Methylene blue extinction.—Sections fixed in formalin or mercurial fixatives were immersed for 24 hours at 25° C. in $5 \times 10^{-4}M$ methylene blue dissolved in veronal acetate buffer at the various pH levels. The sections were examined in water and subsequently mounted in glycerine jelly.

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FIG. 1.—Plasma cells and Russell bodies (arrows) in the lamina propria of the gastric mucosa. Haematoxylin and eosin, × 580.

FIG. 2.—The same tissue as Fig. 1. Lower right, positively-stained mucus in gland cells. P.A.S. celestin blue–haemalum, × 580.

FIG. 3.—Russell bodies in a granuloma (breaking-down sebaceous cyst). P.A.S. celestin blue–haemalum, × 580.

FIG. 4.—Part of Fig. 2 to show detail of Russell body formation. The P.A.S.-positive cytoplasm of some of the plasma cells is well shown. P.A.S. celestin blue–haemalum, × 1,150.
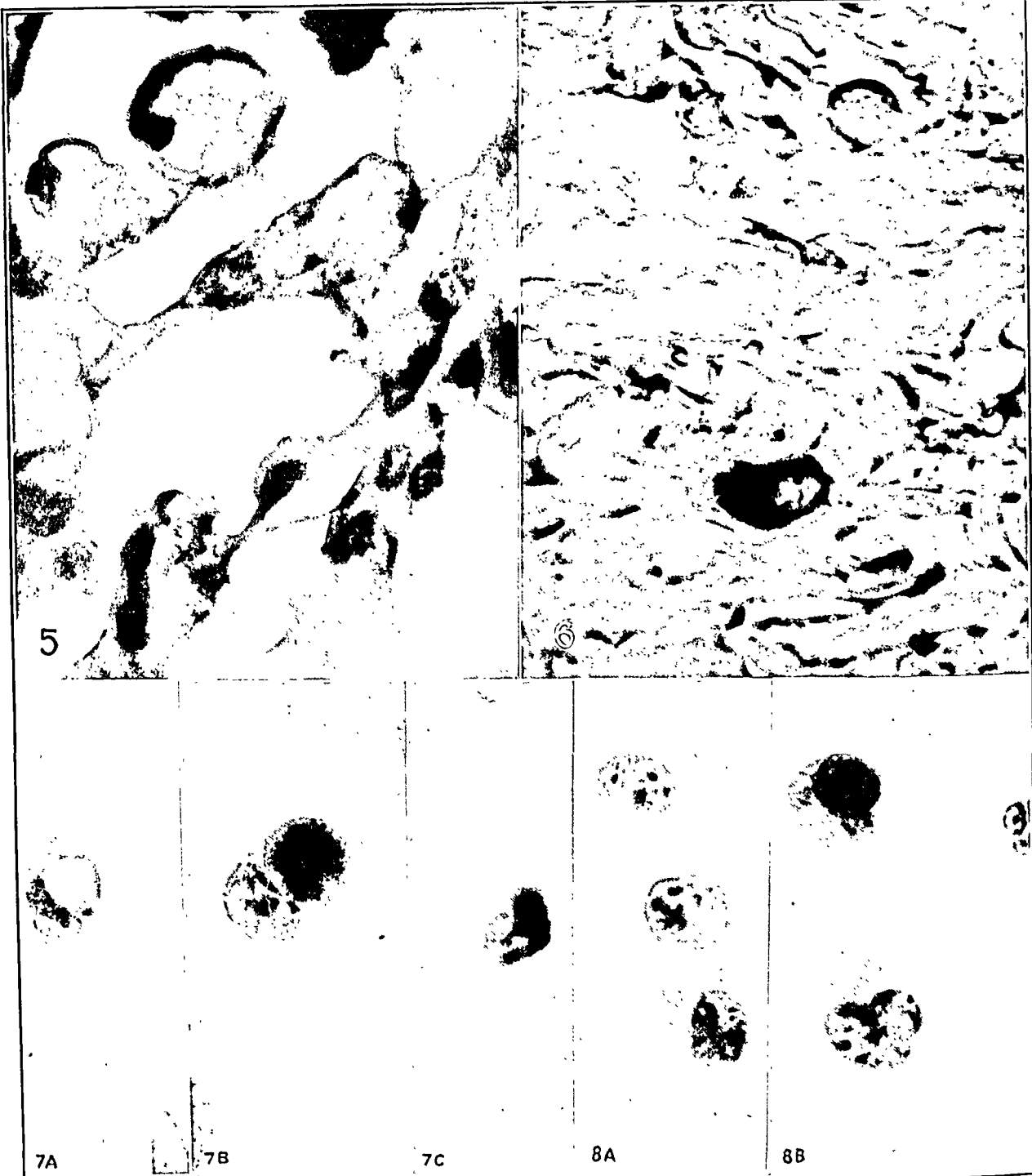
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6

7A   7B   7C   8A   8B
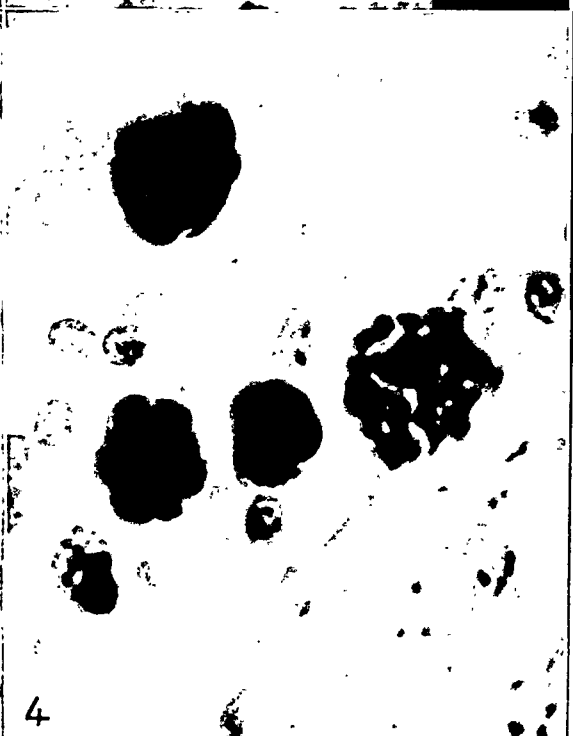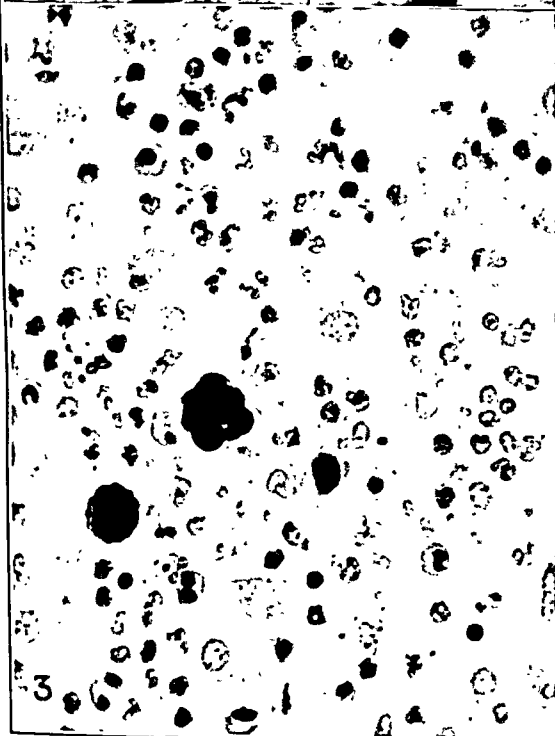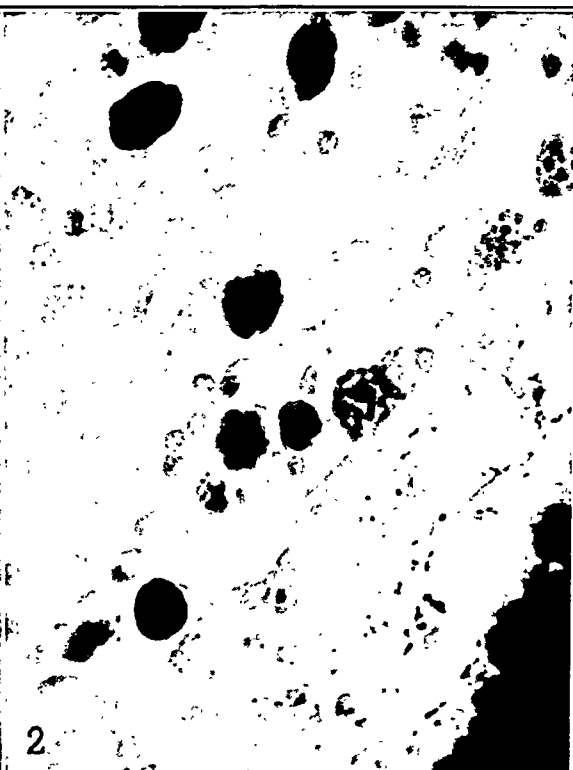
Fig. 5.—Shows the "delicate cementing substance" be-
tween the Russell bodies, stained by pyronin. On
hydrolysis with ribonuclease this disappears. Pyronin-
methyl green, × 1,150.

Fig. 6.—The section is mounted in glycerine jelly, which
accounts for the poor optical qualities of the photo-
graph. Below, red metachromasia shown by tissue
mast cell granules. Above, blue stained Russell bodies
in a plasma cell. 0.25 per cent aqueous thionin,
× 1,150.

Fig. 7a.—Lymphocyte with semilunar nucleus below and
above, pyronin-positive cytoplasm surrounding an
unstained Kurloff body. Pyronin-methyl green,
× 1,150.

Fig. 7b.—Single lymphocyte showing, lower left, blue
staining nucleus; upper right, red P.A.S.-stained

Kurloff body. There is an unstained cytoplasmic halo
around the body in which three greyish dots can be
seen. The darker dots overlying the body are thought
to be precipitated ribonucleic acid. P.A.S. celestin
blue-haemalum, × 1,150.

Fig. 7c.—Below, the nucleus of the lymphocyte. Above,
the Kurloff body. Note the absence of dots seen in
Fig. 7b. P.A.S. celestin blue-haemalum, ×1,150 (after
ribonuclease).

Fig. 8a.—Plasma cells from a case of plasmacytoma show-
ing P.A.S.-positive material in the cytoplasm. P.A.S.
celestin blue-haemalum, × 1,150.

Fig. 8b.—Above, a plasma cell, and, below, a reticulum
cell, from another case of plasmacytoma. Both cells
contain P.A.S.-positive material. P.A.S. celestin blue-
haemalum, × 1,150.

CYSTIC PNEUMATOSIS OF THE LARGE INTESTINE

BY

I. FRIEDMANN

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Cystic pneumatosis of the intestine is a rare condition, but perhaps not so rare as the small number of reported cases suggests. Although recognized since 1876, when Bang first noted gas cysts in the intestine of a woman who had died of volvulus, cystic pneumatosis of the intestine has remained a baffling diagnostic problem.

The condition may be defined as the presence of gas in cyst-like formations in the body. These may be localized in any part of the gastro-intestinal tract ; they have a marked predilection for the ileocaecal region and the duodenum, but are also found in the bladder, vagina, mesentery, parietal peritoneum, and pleura.

Incidence and Distribution.—Cystic pneumatosis is more common in men than in women. It occurs at all ages, but mainly between 25 and 50. About 200 cases have been reported in the literature (Dressler, 1939 ; Sauser-Hall, 1940). Ferrandu in 1935 collected 180 cases, perhaps not all proven. Jackson in 1940 published a survey and added a case of his own, bringing the total of cases in the available literature to 172. Of these, twelve occurred in children and 160 in adults.

Aetiology.—The aetiology of cystic pneumatosis is obscure and none of the theories advanced is entirely convincing. Theories of a neoplastic or a chemical origin of cystic pneumatosis can be disregarded. The clinical picture of the reported cases is also unlike that of a gas-forming infection, the tissue reactions are not suggestive of an inflammatory condition, and bacterial cultures are usually negative. Animal experiments (Sauser-Hall, 1940) have produced no evidence in support of a bacterial origin.

The mechanical hypothesis postulates a break in the intestinal mucosa through which gas is forced into the intestinal wall. The defect can be caused by a localized infection or over-distension of the intestine by gas. There is some evidence in support of this view. In a large proportion of cases cystic pneumatosis is associated with ulcerative diseases of the gastro-intestinal tract, with obstruction and hyperperistalsis—for example, duodenal ulcer and pyloric stenosis, intestinal tuberculosis, chronic ileus caused by adhesions, etc. This theory, however, fails to explain the generalized forms, and the fact that not all people with pyloric stenosis develop cystic pneumatosis. Dressler (1939) suggests that there may be a constitutional weakness in the walls of the lymphatics which enables a comparatively slight rise in the abdominal pressure to cause a dilatation of the lymphatics.

Pathology.—The gross pathology depends on the extent of the gaseous infiltration. As a rule the bowel is covered with grape-like cysts varying in size, shape, and number. They can be localized in all strata of the intestinal wall, usually in the submucosa and in the subserous tissue. Moore (1929) says that in children gas is formed mainly in the mucosa and submucosa, but in adults the gas accumulates in the subserosa. Microscopically the cysts are separated by loose connective tissue lined by endothelial cells resembling the endothelial cells of the lymphatics ; or they may show no lining at all. Giant cells of the foreign-body type are a fairly common feature. The cysts may be empty or may contain some serous mat Haemorrhage, oedema, and eosinophil cells been described in the interstitial tissue

FIG. 1.—Under the intact mucosa the submucosa is transformed into a system of numerous cyst-like cavities resembling emphysema of the lungs. × 20.

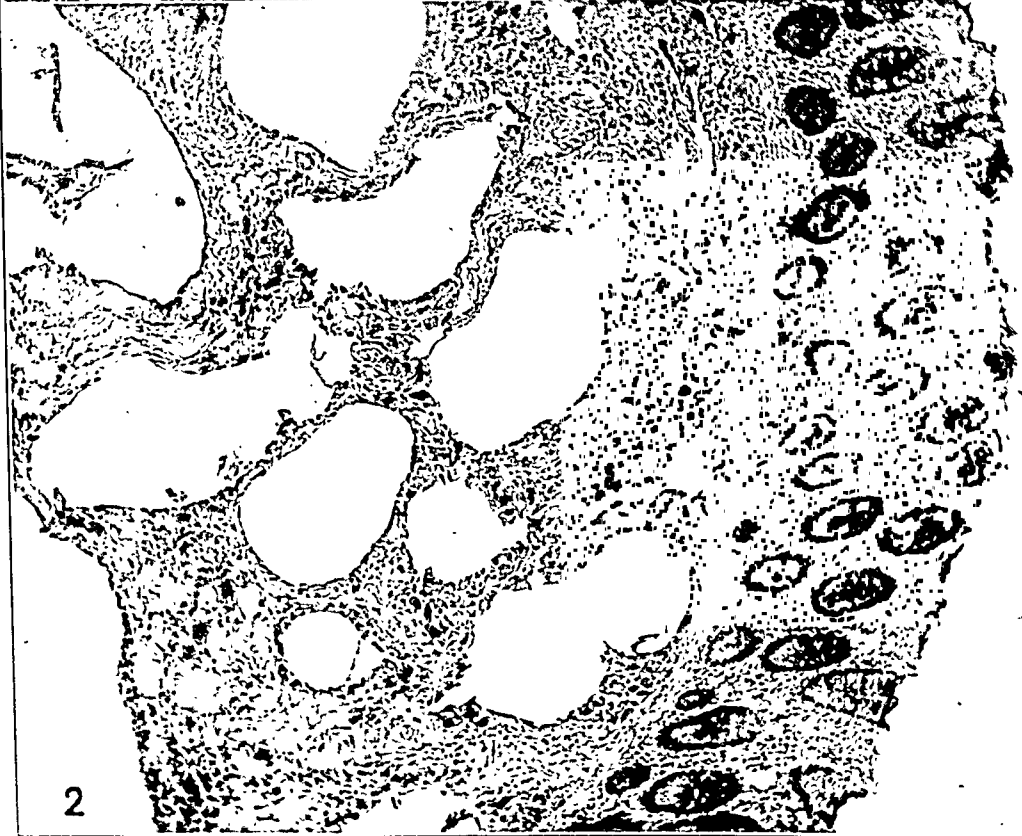FIG. 3.—Shows the cytological details more clearly. × 235.

FIG. 2.—The cavities are lined by endothelial and numerous giant cells of foreign-body type, which can also be seen lying freely in the loose connective tissue separating the cavities. × 80.

Clinical Picture.—Primary cystic pneumatosis unassociated with an organic change in the abdomen is rare (Urban, 1937). According to various authors, in 45 to 75 per cent of all cases reported cystic pneumatosis was associated with a stenosing duodenal ulcer. The clinical diagnosis of cystic pneumatosis is very difficult (Reverdin, 1924), most cases so far reported having been recognized only at operation or at necropsy. Mengis (1938) considers sudden severe abdominal pain the leading clinical symptom of cystic pneumatosis. Recently, however, several cases have been diagnosed by radiography (Lerner and Gazin, 1946; Berglund, 1939).

Cystic pneumatosis of the intestine is in itself a benign process, and the cysts may be very quickly absorbed. Surgical treatment, if necessary, should therefore be directed against the underlying disease, but there is no need for drastic resection of the cystic intestine.

Pelnář (1900) described a case of cystic pneumatosis associated with tuberculosis of the intestine. Barták (1941) reported on the histology of a case of cystic pneumatosis of the caecum which was surgically removed. More recently Vahala (1946) described in some detail another case of cystic pneumatosis of the caecum in which the patient, a 44-year-old physician, was admitted to hospital with a diagnosis of acute appendicitis. He had been suffering for years with occasional bouts of pain in the lower abdomen which came on after exertion but subsided in a few days without treatment. At operation the appendix appeared normal, but the caecum was studded with grape-like cysts in the serosa and deeper in the caecal wall. In this patient the underlying condition was a chronic duodenal ulcer, and the cystic pneumatosis may have been provoked by a mild attack of enteritis.

In the present case, too, the clinical picture was misleading and suggestive of malignant growth of the large intestine.

Case Report

An undernourished woman of 65 was admitted to a provincial hospital with a long history of indigestion and constipation. She had recently been losing much weight. A movable lump, the size of a man's fist, was found on palpation in the ileocaecal region, and malignant growth of the large intestine was suspected. At laparotomy the caecum and ascending colon were found to be transformed into a grape-like mass that showed many partly pedunculated vesicles which collapsed when they were cut open. There was no malignant growth, and the affected parts were resected.

The patient developed bronchopneumonia and died soon after the operation. Necropsy could not be performed.

Histology.—Three small pieces were excised from the ascending colon and fixed in formalin. No bacteriological or chemical examination could be carried out. The macroscopical appearance of the specimens was characteristic: the cut surface showed small round or oval cavities 1 to 3 mm. in diameter situated in the intestinal wall. The consistency of the specimens was sponge-like, not unlike emphysematous lung tissue, and the cavities appeared empty.

Sections stained with haematoxylin and eosin showed the glands to be unchanged and no signs of inflammation in the mucosa (Fig. 1). Closely arranged cystic cavities, varying in size and shape, occupied the whole intestinal wall. They were separated by slightly oedematous connective tissue, and some were lined by fairly large endothelial cells. Others showed flat cells only, or no lining at all. Numerous giant cells of foreign-body type lined some of the cysts; more often they lay freely in the loose connective tissue of the submucosa. Round, small cysts were seen close to the glandular surface, larger ones in the deeper layers. Some of these seemed to have been formed by fusion of two or more smaller cysts under pressure (see the "torn" wall in the larger cyst, Fig. 2). There were a few round cells, but no tuberculous or ulcerative changes and no signs of malignancy.

Most of the cavities were empty. Some contained a faintly blue-staining homogeneous material. In sections stained for fat some red-staining material was seen in a few cysts.

Discussion

This case demonstrates how numerous are the disguises under which cystic pneumatosis of the intestine may occur. As in other cases, there was a long history of dyspepsia and constipation. The exact origin of these signs could not be established. The tumour-like mass was entirely formed by the cystic caecum and ascending colon. The cysts were located in all the layers of the intestinal wall, and they collapsed when incised. They contained some serous and fatty material. This might suggest that the cysts were formed from distended lymphatics. As Lamont (1929) points out, the anatomical arrangement of the lymphatics, which form a complicated series of plexuses, would be quite compatible with the tier-like arrangement of the cysts. Dressler (1939) assumes that the cysts are formed within the lymphatics.

The case was typical in that it simulated another disease—malignant growth—and was only recognized at operation. Its occurrence in an elderly woman was unusual.

1

Summary

A case of cystic pneumatosis of the large intestine in a woman aged 65, simulating symptoms and signs of malignant growth, is described.

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THE SURGICAL PATHOLOGY OF RECTAL CANCER

BY

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The end-results of surgical treatment of rectal cancer are much better than is generally supposed. Expressed in round numbers the following records at St. Mark's Hospital, London, show that almost half the patients who survive the operation of excision of the rectum for cancer are alive after five years. This is particularly satisfactory in view of the fact that surgeons at this hospital adopt a bold and enterprising policy towards rectal cancer, and their operability rate has now reached the astonishing figure of more than 80 per cent. The general operability rate for rectal cancer in other London hospitals has been variously estimated as being below 50 or even below 30 per cent.

The object of this paper is to show how dependent five-year survival rates are upon pathology, especially on the histology of the primary tumour and on the extent of local and lymphatic spread. The present analysis is based on 716 cases treated by radical excision between 1928 and 1941 inclusive. Only twelve of this series of 716 patients could not be traced for a period of five years. These have been regarded as having died, which is weighting the scales unfavourably, but being few in number their effect either way is slight. No distinction has been made between deaths due to recurrence and those due to other causes. Therefore the figures are true survival rates: they record only the number of operation survivors still alive after five years.

The general results of the surgical treatment of rectal cancer at St. Mark's Hospital in the years 1928 to 1941 inclusive were that 337 out of 716 patients were alive after five years—that is 41.7 per cent (Table I). Approximately half these patients were treated by perineal and half by a combined operation. In cases without lymphatic metastases the percentage of five-year survivors was approximately the same whatever the operation, but for patients with lymphatic metastases the combined operation results were approximately 16 per cent better than those for perineal excision.

No doubt there are many factors which influence the end-results of the surgical treatment of rectal cancer, such as the skill of the surgeon, the nursing facilities available, the after-treatment, and also social and individual factors which need not be mentioned, but in a large series of cases like this it is easy to show that the chief factor affecting five-year survival rates is the pathology of the disease at the time of operation, especially the extent of local, venous, and lymphatic spread.

Histological Classification of Rectal Cancer

According to its histology, rectal cancer can be divided into three main groups—adenocarcinoma, colloid carcinoma, and carcinoma simplex. Each of these has a distinctive histological pattern which is of significance in relation to prognosis. The chief features of each group are given in the following paragraphs.

Adenocarcinoma (non-mucinous columnar-celled carcinoma).—This growth is composed of columnar or cubical cells arranged for the most part in a tubular or acinar pattern surrounding glandular spaces which may be irregular in shape and either empty or partly filled with cellular debris. The distinctive feature of this variety of carcinoma is that there is no sign of mucus secretion either in the cells or glandular spaces. This is the commonest variety of rectal cancer. It may be further subdivided according to the degree of differentiation of the tumour cells into three subgroups: (1) low grade of malignancy, (2) average, and (3) high grade of malignancy. Follow-up records show that approximately 60 per cent of the patients marked low grade have survived five years, whereas less than 30 per cent of the patients marked high grade have lived for this period after operation. One reason for the influence of histology on prognosis becomes obvious when we compare the incidence of lymphatic metastases in various grades.

TABLE I

RESULTS OF SURGICAL TREATMENT OF RECTAL CANCER*

Opera-tion survivals	Untraced (regarded as dead)	Died in less than 5 years (from any cause)	Alive at 5 years	% of 5-year survivals
716	12	367	337	47.1

* All cases operated on at St. Mark's Hospital 1928 to 1941 inclusive (operation deaths excluded).

TABLE II
RELATIONSHIP OF HISTOLOGICAL GRADES TO INCIDENCE OF
LYMPHATIC METASTASES (1,807 CASES)

Grade of tumour	Total cases	Cases with metastases	% with metastases
Low grade ..	190	35	18.4
Average grade ..	1162	515	44.3
High grade ..	455	356	78.2

The figures for 1,807 cases of rectal cancer are set out in Table II, from which it will be seen that metastases were found in only 18.4 per cent of " low grade " tumours but were present in 78.2 per cent of " high grade " tumours.

Colloid carcinoma (mucoid, or mucus-secreting carcinoma).—This has a similar basic structure to adenocarcinoma but differs in that mucus is secreted. When stored in individual cells the mucus gives rise to a " signet ring " appearance, but if secreted into glandular spaces the epithelial cells often seem to be relatively few in number, being found only at the margin or floating in the secretion. The secretion is not actually " colloid," but the tumour has long been known as a " colloid carcinoma," and it would be confusing to abandon this name. Three histological grades of malignancy may be distinguished in this tumour also, though the grading is not so easy nor the prognostic significance so clear-cut as with non-mucinous adenocarcinoma. Colloid carcinoma is the second commonest variety of rectal cancer, composing about 20 per cent of all cases.

Carcinoma simplex (anaplastic polygonal-celled carcinoma).—This term is used to describe an anaplastic type of carcinoma composed of polygonal or spheroidal cells destitute of any glandular arrangement and not showing any mucus secretion. The tumour cells are scattered about singly or in small clusters, or they may be arranged in a solid trabecular or alveolar pattern. It may be difficult at first to decide whether the growth is a sarcoma or a carcinoma, but some part of the tumour generally has features characteristic of carcinoma. These anaplastic tumours show a special tendency towards metaplasia, and though the growth has originated in columnar epithelium there may be regions resembling squamous-cell or transitional-cell carcinoma. These tumours are all of a high grade of malignancy, have generally caused widespread lymphatic metastases, and have a bad prognosis.

Influence of Local Spread

The extent of local spread in rectal cancer can often be decided by cutting a thin slice with a sharp knife through the region in which there appears to be deepest penetration into the bowel wall or surrounding tissues. The boundaries of local spread are most clearly seen when a malignant tumour begins to spread into the fatty tissue. The final decision as to extent of spread must of course depend on the result of the microscopic examination of sections, but the only cases in which there is likely to be much discrepancy are septic growths or highly anaplastic tumours, or those cases in which there is an abscess at the margin, or fibrosis is found following infection or radiation.

It is useful to have a simple method of recording the extent of local spread and of correlating this with lymphatic or venous spread. The system we have used at St. Mark's Hospital for many years is to classify patients who have been treated by radical excision into four groups according to the extent of spread of the disease as revealed by the examination of the operation specimen. In the first or initial stage the growth is limited to the bowel, and the patient at this stage is described as an " A " case. The prognosis after surgical treatment of such cases is excellent. The second stage is reached when the malignant tumour has spread by direct continuity into adjoining structures but not yet given rise to lymphatic metastases. At this stage a patient is described as a " B " case, and experience has shown that such patients also have a relatively good prognosis after surgical treatment. The third stage is reached when the malignant growth has spread still further and given rise to lymphatic metastases. Patients at this stage are known as " C " cases. If the malignant growth has spread to distant organs such as the liver, this is described as the fourth stage.

The examination of operation specimens of rectal cancer has shown that in about 15 per cent of cases the growth is still at the first stage and confined to the rectum only. In approximately 35 per cent there is some local extra-rectal spread but no lymphatic metastases, and in the remainder the tumour has reached a later stage and lymphatic metastases are present. These proportions have remained fairly constant from year to year though varying slightly with the operability rates at different periods. The influence of local spread can be shown in a very convincing way by comparing the prospects of patients in the first and second stages of the disease—the so-called A and B cases. Between 80 and 90 per cent of A patients have survived five years, but only 60 to 70 per cent of the B cases.

Influence of Venous Spread

When operation specimens of intestinal cancer are dissected, evidence of extension within veins may be found in 10 to 15 per cent of cases, and this usually assumes the form of a solid cord extending a short distance only. The intravascular growth preserves its continuity with the primary tumour and is no more than a special form of direct local extension. It is as if the malignant tumour, having found a path of least resistance, has pushed a root-like process along the lumen of the vein. This is much the commonest manifestation of venous spread, but occasionally one finds also a massive permeation of the haemorrhoidal veins accompanied by thrombosis. In both types of venous spread the growth within the veins has obviously been derived from the primary tumour and still remains in contact with it. Veins may also be secondarily invaded from neighbouring lymphatic metastases, but evidence is rare.

Intravascular spread is most often found in anaplastic varieties of carcinoma, being present in more than 30 per cent of tumours reported as high-grade but in less than 3 per cent of those marked low-grade of malignancy. The veins are seldom invaded until the tumour has spread by direct continuity through the wall of the bowel.

It is not easy to express the prognostic significance of venous spread. The presence of carcinoma cells within the veins of an operation specimen would seem at first to be of very sinister significance, but it has not proved to be as bad as might be expected. All that can be said is that the finding of clumps of carcinoma cells within a vein certainly makes it more likely but does not definitely prove that spread to the liver or other viscera has already taken place. Post-mortem examinations on patients who have died soon after operation for rectal cancer have shown that extension to the liver has occurred in only half the cases in which the dissection of the operation specimen had shown involvement of veins. On the other hand hepatic metastases have been found when the examination of the operation specimen failed to reveal any evidence of intravenous extension. We must remember there is this fundamental difference between venous and lymphatic spread. Lymphatic metastases remain fixed in the glands, and if lymphatic spread has occurred it leaves a permanent footprint, so to speak. Venous emboli on the other hand may leave no track behind them.

Concealed and unsuspected hepatic metastases account for many deaths in the first year or two after operation for intestinal cancer. On the other hand there is ample evidence that patients may remain in good health for many months or even years in spite of hepatic metastases.

Influence of Lymphatic Spread

Success or failure in the surgical treatment of rectal cancer depends very largely on the extent of lymphatic spread. Therefore no pathological report on an operation specimen is complete without examination of the lymphatic glands, and in rectal cancer it adds greatly to the value of a report if the position of metastases is recorded. To do this the glands should be dissected out from the perirectal fat, a scale drawing being made at the same time to indicate their position. The glands are then numbered and blocked in groups. After microscopic examination the position of metastases is marked on the diagram by inking in the affected glands. An alternative method is to remove the glands in two groups: (1) those in the immediate vicinity of the tumour and (2) the topmost glands situated where the vascular pedicle is ligatured. If an operation specimen is dealt with in this way it is possible to say not only how many glands contain metastases but also where these metastases were situated. Our practice at St. Mark's Hospital is to attach to the report two photographs, one of the operation specimen showing the position and size of the primary tumour, the other a photograph of a scale drawing made after a dissection of the glands and blood vessels, and in this the position of the primary tumour is indicated by shading and the extent of local spread by stippling. Glands and blood vessels not containing growth are merely outlined, but cancerous deposits are recorded in black.

The dissection of operation specimens of rectal cancer has shown that the first glands to receive metastases are almost invariably those lying nearest to the primary tumour. In the case of growths of the lower third and ampulla of the rectum the first metastases are found in the pararectal glands on the same level with or immediately above the primary tumour. The next glands to be affected are the superior haemorrhoidal, which are usually invaded in sequence from below upwards. In an advanced case of cancer of the lower third or ampulla the metastases come to form an unbroken chain extending from the regional group of glands to those situated at the point of ligature of the inferior mesenteric vessels. Occasionally a case is met with in which a metastasis is present high up in the chain of haemorrhoidal glands though those at a lower level are free from deposits. This may be due to an anomaly whereby some lymphatic trunks by-pass glands *en route* and pass

directly upwards to reach the glands in the recto-sigmoid region.

The general direction of lymphatic spread for growths situated in the upper third of the rectum and recto-sigmoid region is also upwards, and it is very rare to find metastases in glands below the primary tumour, though this may occur if the upward spread is blocked. Downward spread of this character has been met with in less than 1 per cent of cases. Evidence of lateral lymphatic spread may sometimes be seen in tumours situated in the lower third of the rectum, but even in these situations it would seem that the upward path is the main highway of lymphatic drainage and is the one most commonly taken by cancerous emboli.

The overwhelming influence on prognosis of lymphatic spread in rectal cancer is shown by the fact that the five-year survival rate at St. Mark's Hospital for cases with lymphatic metastases has been only 26.2 per cent whereas 68.1 per cent of the patients without metastases have survived five years (Table III). These figures are for all types of radical excision, but when a comparison is made of the results of perineal and combined operations the unfavourable effect of lymphatic metastases is seen to be much more pronounced after perineal than after combined excision. The five-year survival rate of perineal excision in cases with lymphatic metastases has been only 17.4 per cent compared with the 33.3 per cent for the combined operation (Table IV). The reason for this is because metastases in the upper haemorrhoidal glands may be removed by a combined though not by a perineal excision.

Since survival after operation depends so much on the position of metastases, we have adopted the plan of dividing patients with metastases (so-called C cases) into two groups. A case is

TABLE III
INFLUENCE OF LYMPHATIC SPREAD ON FIVE-YEAR SURVIVAL RATE AFTER EXCISION OF THE RECTUM*

	Operation survivals	Alive at 5 years	% of 5-year survivals
Cases without lymphatic metastases (A and B)	357	243	68.1
Cases with lymphatic metastases (C)	359	94	26.2

* Based on all cases operated on at St. Mark's Hospital 1928 to 1941 inclusive.

described as C1 if the regional glands alone contain metastases, whereas if there is more extensive lymphatic spread involving also the glands at the point of ligature of the blood vessels the case is classified as C2. This has proved useful because patients with only a few regional lymphatic metastases have a moderately good prognosis after radical surgical treatment, but with more extensive lymphatic spread the prospects of cure are not so good. Obviously both the position and number of metastases is important. Patients with less than four or five lymphatic metastases fairly often survive five years, but this fifth anniversary is rarely reached by those with more than five metastases. So here in conclusion is a simple generalization with regard to rectal cancer which it is easy to remember: patients with five or more metastases rarely live for five years.

Summary

A pathological report on a case of rectal cancer should include a description of the histology of the growth and an estimate of the extent of local venous and lymphatic spread. These pathological features are all interrelated, but each also has its own significance in relation to prognosis.

TABLE IV
COMPARISON OF FIVE-YEAR SURVIVAL RATES AFTER PERINEAL AND COMBINED EXCISION*

	Group	Operation survivals	Untraced (regarded as dead)	Died in less than 5 years (from any cause)	Alive at 5 years	% of 5-year survivals
Perineal excision	A	59 (16.8%)	2	10	47	79.7
	B	130 (37.1%)	3	48	79	60.8
	C	161 (46.1%)	3	130	28	17.4
	Total	350	8	188	154	44.0
Combined excision	A	46 (12.6%)	—	7	39	84.8
	B	122 (33.3%)	1	43	78	63.9
	C	198 (54.1%)	3	129	66	33.3
	Total	366	4	179	183	50.0

* St. Mark's Hospital cases 1928 to 1941 inclusive (operation deaths excluded).

THE IRON RESERVE OF A NORMAL MAN

BY

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No one seems to have attempted to measure the reserve of iron available for haemoglobin synthesis in man. Hahn (1937) estimated that 57 per cent of the total iron in a dog was contained in the blood haemoglobin, 20 per cent was stored in a form potentially available for haemoglobin synthesis, and 23 per cent was bound in myohaemoglobin, cell enzymes, etc., and was not available for haemoglobin formation. The available iron reserve of the dog was thus sufficient to replace about one-third of its circulating haemoglobin, and clinical experience of unassisted recovery from acute haemorrhage suggests a similar figure in man.

The rate of haemoglobin regeneration after haemorrhage depends upon the stimulus to and response of the marrow as long as iron is freely available. A normal man can draw upon his iron reserve as well as upon dietary iron for blood regeneration after acute haemorrhage, but if his iron reserve is depleted by repeated haemorrhage the rate of haemoglobin regeneration is limited by the dietary iron available. Thus if a man is kept anaemic by repeated bleeding, his rate of haemoglobin regeneration will fall progressively until, when his iron reserve is exhausted, a constant level is attained. This principle has been used in this experiment to calculate the size of the available iron reserve of a normal man.

Material and Methods

The subject was a man 37 years old weighing 63 kg. He had lived on the normal English civilian diet since his discharge from the Army sixteen months before the experiment began.

He was bled every weekday an hour and a half after breakfast, having sat down for 30 minutes before the venepuncture. Anaemia was established by two blood donations of 800 and 900 ml. respectively. The amount of blood removed daily for routine tests was at different times, 5.5, 6.5, and 20 ml., as indicated in Figs. 1 and 2. In all, 2,750 ml. of blood were removed during the 136 days of the experiment.

The vein for venepuncture was not compressed in any way. Wintrobe's dry oxalate mixture was used as anticoagulant, and pipettes were filled immediately, before sedimentation had begun. All estimations were made in duplicate. Haemoglobin was estimated photo-electrically as oxyhaemoglobin. The optical density of a 1/201 dilution of blood in 0.4 per cent ammonia was measured in a Spekker absorptiometer calibrated against iron determinations on washed red cells, with a suitable correction for the plasma content of whole blood. Red cell counts, Wintrobe haematocrit determinations, and reticulocyte counts were made in the usual way. Serum and plasma specific gravities were estimated by the copper sulphate specific gravity technique of Phillips and van Slyke (1945).

The daily rate of haemoglobin regeneration was calculated from the best-fitting polynomials to the observed haemoglobin levels in each period of the experiment.

Results

The course of the blood haemoglobin level is shown in Figs. 1 and 2. (No blood was taken during the interval of nine days between the two charts.) It will be observed that there was a relatively wide day-to-day fluctuation in the haemoglobin level. This was closely paralleled by fluctuations in the haematocrit value and serum specific gravity, showing that even under the standard conditions of the experiment there was a day-to-day variation of about ± 5 per cent in the plasma volume. These random fluctuations were excluded from the calculation of haemoglobin regeneration by the statistical fitting of curves to the data.

The falls in haemoglobin level after the two large venesections corresponded to a blood volume of 5.3 and 5.5 litres respectively, in good agreement with a value of 5.45 litres previously obtained by the Evans blue technique. It will be observed that the haemoglobin level did not reach its nadir for a few days after the venesection; evidently the compensatory increase in plasma volume occupied this interval.

If the blood volume and amount of blood withdrawn daily are known, it is easy to calculate the
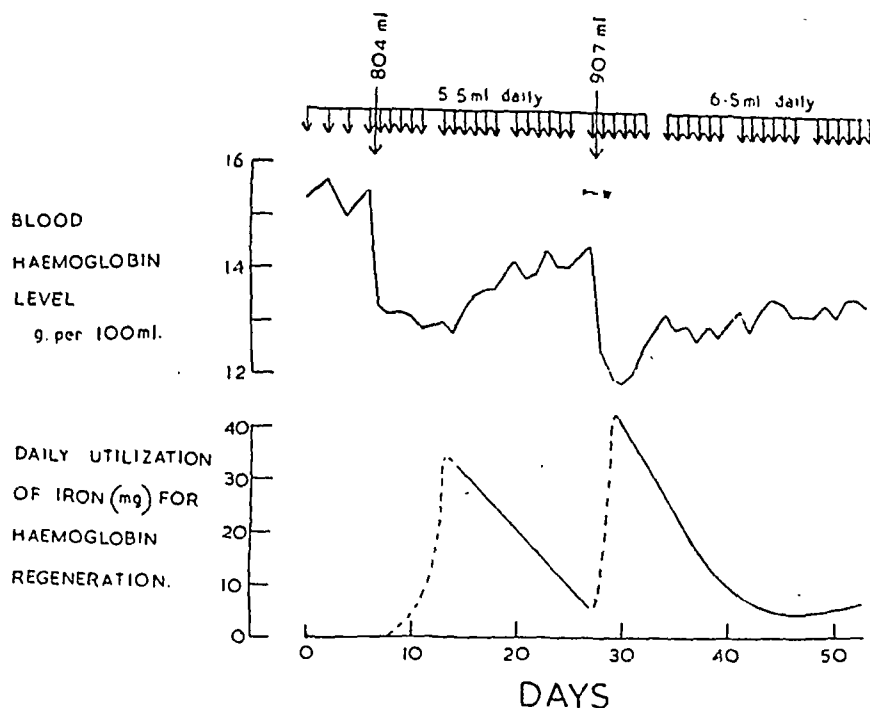
BLOOD REMOVED

804 ml

907 ml

5 5 ml daily

6·5 ml daily

BLOOD
HAEMOGLOBIN
LEVEL
g. per 100ml.

DAILY UTILIZATION
OF IRON (mg) FOR
HAEMOGLOBIN
REGENERATION.

DAYS

FIG. 1.—Blood haemoglobin levels during repeated bleeding.

We may deduce that the subject's iron reserve was finally exhausted at this time.

The size of the available iron reserve was calculated as follows: The initial blood volume at day 6 was 5.3 litres, the haemoglobin level 15.2 g. per 100 ml., and the total circulating haemoglobin iron $53 \times 15.2 \times 3.34 = 2,690$ mg. The final blood volume at day 125 was 5.7 litres (see below), the haemoglobin level 13.2 g. per 100 ml., and the total circulating haemoglobin iron $57 \times 13.2 \times 3.34 = 2,510$ mg.

A total of 1,190 mg. of iron was removed by venepuncture during the interval. We therefore have the equation:

$$R + 2,690 - 1,190 + x = 2,510$$
$$\text{or } R = 1,010 - x \ldots\ldots\ldots (1)$$

where R is the initial iron reserve in mg., and x the total iron absorbed and retained from the food.

daily utilization of iron for haemoglobin regeneration in excess of the normal replacement of effete red cells. The lower curves on Figs. 1 and 2 show this value, which fell to a minimum on day 45 and thereafter remained constant.

We may obtain an estimate of x by considering the periods from days 62 to 102 and 104 to 123 inclusive, when the iron reserves were completely exhausted and iron absorption from the food was

BLOOD REMOVED

6·5 ml. daily

20ml daily

Fe SO₄ 1·65g/daily

BLOOD
HAEMOGLOBIN
LEVEL
g. per 100ml.

DAILY UTILIZATION
OF IRON (mg) FOR
HAEMOGLOBIN
REGENERATION.

DAYS

FIG. 2.—Response of blood haemoglobin to repeated bleeding after exhaustion of the iron reserve.

maximal. In the first of these periods, when 6.5 ml. of blood were removed daily, the haemoglobin level was increasing at the rate of 0.0844 g./litre/day, and the iron utilized daily for this increase amounted to 0.0844 × 3.34 × V mg., where V is the blood volume in litres. The blood removed during these 41 days contained 104 mg. of iron. If, therefore, F mg. is the mean daily amount of iron utilized for haemoglobin regeneration in excess of the normal replacement of effete red cells, we have:

$$F - \frac{104}{41} = 0.0844 \times 3.34V \quad \ldots\ldots\ldots(2)$$

From days 104–123, when 20 ml. of blood were removed daily, the haemoglobin level fell at the rate of 0.2075 g./litre/day ; 161 mg. of iron was withdrawn in blood in the 20 days, and consequently:

$$F - \frac{161}{20} = -0.2075 \times 3.34V \quad \ldots\ldots\ldots(3)$$

Combining equations (2) and (3) we have:
$$V = 5.7 \text{ litres} \qquad F = 4.1 \text{ mg.}$$

We may assume that the iron absorption was maximal from day 46, when the iron reserves were exhausted, so that the minimum value to be assigned to x in equation (1) is $80 \times 4.1 = 328$ mg. The value of F in the first part of the experiment remains unknown, since it rose from zero on day 6, when the blood count was normal, to a maximum value of 4.1 on or before day 46. If we take F as zero for these 40 days, x in equation

(1) will be underestimated as 328 mg., whereas if we take F as 4.1 mg. throughout the experiment, x will be overestimated as 492 mg. The iron reserves therefore lie between:

$$R = 1,010 - 328 = 682 \text{ mg. and } R = 1,010 - 492 = 518 \text{ mg.}$$

The intermediate value of 600 mg. may be taken as a reasonable approximation.

Discussion

This experiment would have required much more stringent control of the dietary iron intake but for the fortunate fact that our present rationing system does not permit any substantial variation of the diet. It is thus reasonable to assume that the iron intake was the same over any period of two or three weeks during the course of the experiment.

The conclusions drawn from this experiment are based upon the assumption that the supply of iron was the limiting factor in blood formation. The marrow must normally replace effete red cells at a rate corresponding to the production of 45 ml. of blood daily, and the loss of a further 6 or 7 ml. daily would not impose any great strain if the supply of raw materials were adequate. Moreover, it is common experience that the daily increase in the red cell count in the cure of anaemia corresponds to the production of 15 g. of haemoglobin a day, and only 0.9 g. was withdrawn daily during most of this experiment. Even the final withdrawal rate of 2.5 g. of haemoglobin
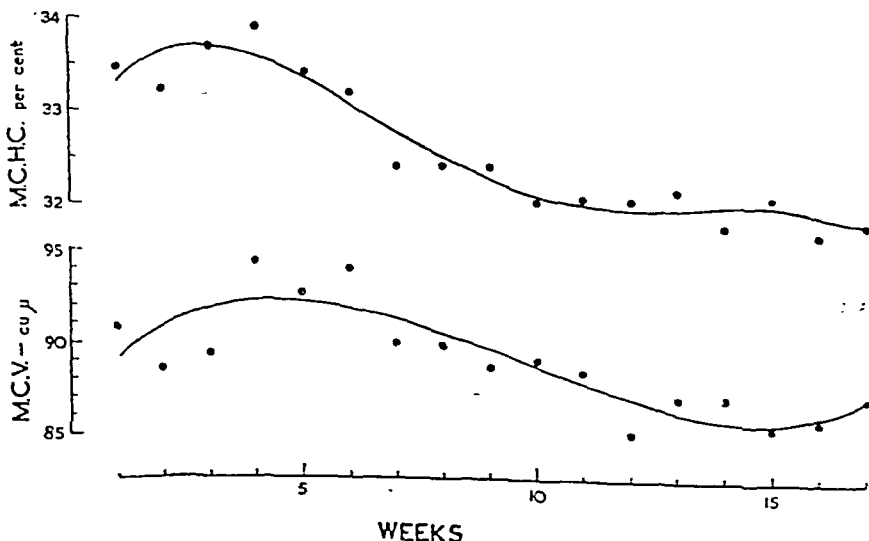

FIG. 3.—The decline in mean corpuscular volume and haemoglobin concentration with repeated bleeding

a day would scarcely strain the capacity of a marrow already made hypertrophic by 96 days of anaemia. The supply of accessory factors would presumably be adequate, for nutritional macrocytic anaemia is rare in this country.

These assumptions are confirmed by the fact that both the mean corpuscular haemoglobin concentration and mean corpuscular volume after a preliminary rise fell steadily during the experiment (Fig. 3). Both the rise and the subsequent fall were statistically significant, for the data could only be satisfactorily fitted by cubic and quartic curves respectively. The final proof, however, was when, after day 123, the daily withdrawal of 20 ml. of blood was maintained, but full doses of iron were given by mouth. After a short interval the rate of haemoglobin regeneration rose steeply.

It is sometimes believed that iron-deficiency anaemia due to haemorrhage is accompanied by a reticulocytosis which serves to distinguish it from other forms of hypochromic anaemia. This view was not, however, borne out by this experiment. The reticulocyte count did rise from 0.2 per cent at the beginning of the experiment to 0.4 per cent when the rate of haemoglobin regeneration was maximal, but it fell again thereafter, and was only 0.1 per cent when 20 ml. of blood was being withdrawn daily and the haemoglobin level was falling. When iron was administered the reticulocyte count rose to 0.7 per cent.

The estimated value of 600 mg. for the iron reserve available for haemoglobin synthesis was sufficient to replace about one-fifth of the subject's red cells. The relative smallness of this reserve emphasizes the importance of iron therapy after any substantial haemorrhage.

The experiment further showed that the present British diet can afford some 4 mg. of iron a day for haemoglobin synthesis, in addition to the 1 mg. or so needed daily to replace iron excreted in the bile and urine (Hynes, 1948). The subject's estimated total iron intake was of the order of 15 mg. a day, so that about one-third of this total was physiologically available for absorption and haemoglobin synthesis.

This potential dietary surplus of 4 mg. of iron a day is a very small margin—it would be balanced by a loss of only 8 ml. of blood a day. The body must depend almost entirely on its available iron reserve for rapid recovery from haemorrhage, for the dietary iron suffices for a haemoglobin increase of only 0.15 g. per 100 ml. per week.

There is still some doubt as to the safe interval between blood donations for transfusion. If, as is usual, 450 ml. of blood is taken from a normal man, it will contain some 240 mg. of iron. This amount will be rapidly taken from the available reserve to reconstitute the haemoglobin level, whilst the reserve will be more gradually replenished from the dietary iron. If iron absorption were immediately maximal the process would take 60 days. A normal woman requires about 1 mg. of iron a day to replace menstrual losses, so that the replacement of 220 mg. of iron from a blood donation of 450 ml. would require 73 days if iron absorption were maximal from the beginning. Thus a cumulative iron deficiency would not follow even in normal women, unless blood were donated more than five times a year. Even moderate menorrhagia, however, might of itself cause iron losses equivalent to more than 4 mg. daily.

Summary

1. The iron reserve of a normal man was estimated by bleeding him until his rate of haemoglobin regeneration fell to a constant level. The daily absorption of iron from the food, in excess of the normal requirement to replace effete red cells, was calculated from the different rates of haemoglobin regeneration when 6.5 and 20 ml. of blood respectively were withdrawn daily after exhaustion of the iron reserve.

2. The iron reserve was thus calculated to be about 600 mg.

3. It was calculated that the present English diet can yield 4 mg. of iron daily for haemoglobin synthesis in addition to the normal needs for replacing effete red cells.

4. This extra 4 mg. of iron would replace a loss of only 8 ml. of blood a day by an ordinary man.

5. The iron reserve would not be depleted by blood donations of 450 ml. as often as five times yearly by a man or by a woman with normal menstrual loss. Moderate menorrhagia, however, could of itself demand more than the available 4 mg. of extra iron.

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ELUTION OF AN INCOMPLETE TYPE OF ANTIBODY FROM THE ERYTHROCYTES IN ACQUIRED HAEMOLYTIC ANAEMIA

BY

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A notable advance in the study of the haemolytic anaemias was made by Boorman, Dodd, and Loutit (1946), who showed that it was possible to differentiate between the congenital and acquired types of acholuric jaundice by submitting the washed erythrocytes from these patients to the action of an anti-human globulin serum (Coombs and others, 1945). The erythrocytes of 17 patients with congenital familial type were not agglutinated under these conditions, whereas those from seven patients with clinically acquired type were all agglutinated and so presumably had antibody globulin adsorbed to their surface. No free antibody was found in the serum from these cases, probably because all circulating antibody was fixed on the erythrocytes.

During a personal study of six patients with acquired haemolytic anaemia the washed erythrocytes of all cases were found to agglutinate strongly with an anti-human globulin serum. Corresponding antibody of the "incomplete" type (Race, 1944) was not, however, found free in the serum of any of these cases, although cold agglutinin of low titre was present in the serum of two of them.

In order to elucidate further the nature of the antibody adsorbed upon the erythrocyte in these patients it seemed necessary to attempt to dissociate the antibody from its union with the erythrocyte. After a number of tentative experiments a satisfactory technique for this purpose was elaborated, and the results presented provide direct evidence that an antibody of "incomplete" type is adsorbed on the erythrocytes in these cases. Some *in vitro* properties of this antibody were also investigated.

Materials and Methods

A brief summary of the pertinent clinical and haematological findings in the patients studied is given in Table I. Venous blood samples were obtained with a dry syringe and immediately defibrinated by stirring with a glass rod. Samples were examined before and after splenectomy in Case 4. In the remaining cases all the samples examined were collected at varying intervals after splenectomy had been performed.

TABLE I

MAIN CLINICAL AND HAEMATOLOGICAL FINDINGS IN SIX CASES OF ACQUIRED HAEMOLYTIC ANAEMIA

Case	Age	Sex	Red cells 10⁵ per c.mm.	Haemo-globin per cent (Haldane)	Reticulo-cytes per cent	Serum bilirubin mg. per 100 ml.	Osmotic fragility	Auto-agglu-tination	Direct Race – Coombs test	Treat-ment
1	74	M	2.8	60	30.0	3.2	Slightly raised	Absent	+++	Spl.
2	33	M		30			Normal	Absent	+++	Spl.
3	66	F	1.66	36	6.4	3.6	Normal	Present (trace)	+++	Spl.
4	43	F	1.0	32	18.0	3.6	Normal	Present (marked)	+++	Spl.
5	46	F	2.85	71	11.8	2.0	Normal	Absent	+++	Spl.
6	28	M	2.5	52	15.0	2.8	Normal	Absent	+++	Spl.

Spl. = Splenectomy

Anti-human globulin serum.—Rabbits were immunized with two doses of the globulin precipitate from 7.5 ml. of Group O human serum dialysed against distilled water for forty-eight hours, followed by two doses of 1 ml. of whole serum. The injections were given into the ear vein at five-day intervals and the animals bled seven to ten days after the last injection. After suitable adsorption with a mixture of packed washed human erythrocytes of Groups A, B, and O, the working dilution of the serum was found by testing it against erythrocytes sensitized with a serum containing incomplete anti-Rh antibody. A dilution of 1/20 to 1/40 gave rapid agglutination of the test cells at 37° C.

pH measurements.—These were made with the glass electrode unless otherwise stated.

Citrate–HCl buffer M/10 (Sörensen).—pH 3.2 to 3.4. This was prepared according to the directions given by Clark (1928).

Elution of antibody.—This was effected by a modification of the procedure used by Landsteiner and van der Scheer (1936) to dissociate azostromata-antibody complexes. The dilute acetic acid used by these workers was replaced by M/10 citrate-HCl buffer at pH 3.2 to 3.4. The procedure adopted was as follows. 20–50 ml. of whole blood was centrifuged, the plasma removed, and the erythrocytes washed three times with physiological saline. An equal volume of distilled water was then added to the packed cells and the mixture lysed by repeated freezing and thawing. Erythrocyte stroma was then prepared according to the principle of Jorpes (1932). The lysed blood was diluted with a further five volumes of distilled water and the stroma precipitated by adjustment of the mixture to pH 5.6 to 5.8 by addition of the minimal necessary quantity of N HCl. The precipitate removed by gentle centrifugation was then freed as far as possible of haemoglobin by repeated washing with M/15 phosphate buffer at pH 5.6 to 5.8. To one volume of washed packed stromata was then added 2 to 3 volumes of the citrate-HCl buffer, and after thorough mixing the

pH was readjusted, if necessary, to between pH 3.2 and 3.4 by the addition of N/1 HCl. After leaving the mixture for 10 or 15 minutes at room temperature the stromata were separated by centrifugation, and the supernatant removed and rapidly adjusted to pH 7.2 to 7.4 by the dropwise addition of 5N NaOH, using phenol red as an external indicator. During neutralization a heavy precipitate of stroma protein separated, carrying down with it most of the residual colour in the solution. This precipitate was removed and the supernatant eluate, now practically colourless or faintly tinged with the brown colour of acid haematin, was stored in the frozen state in screw-capped bottles over dry carbon dioxide ice.

Testing of the eluates.—Serial dilutions were made in physiological saline and two drops of each dilution mixed with two drops of a 2 per cent suspension of washed human erythrocytes in small test tubes. In comparative tests of titre the erythrocytes of the same Group O donor were used throughout.

After incubation for one hour at 37° C. the erythrocytes were washed twice with physiological saline and resuspended to approximately 2 per cent concentration, and then two drops of a suitably diluted anti-human globulin serum added to each tube. After a further 30 minutes in the incubator the tests were read by gently shaking each tube, and the results recorded on a rough quantitative scale:

　　+ + + = Large compact clumps
　　+ = Clumps just visible to the naked eye

A control of washed 2 per cent unsensitized cells plus two drops of anti-human globulin serum was included in each test.

Results

Range of erythrocytes affected.—The eluates have been found to react with all human erythrocytes tested ; that is to say exposure of the erythrocytes to the eluate results in the " coating " of the erythrocytes with an antibody so that the cells are rendered agglutinable after washing and addition

TABLE II

EFFECT OF ELUATES ON VARIOUS TYPES OF ERYTHROCYTES

(1 vol. eluate + 1 vol. 2 per cent erythrocyte suspension incubated for 1 hour at 37° C. Cells washed three times with saline. Anti-human globulin serum added.)

Source of eluate	Blood group of human erythrocytes				Rhesus monkey 1	Rhesus monkey 2	Mouse	Guinea pig	Rabbit	Rat	Fowl	Horse	Sheep
	A	B	ORh+	ORh′–									
Case 1	+ + +	+ + +	+ + +	+ + +	+	+	—	—	—	—	—	—	—
,, 2	+ + +	+ + +	+ + +	+ + +	+	+	—	—	—	—	—	—	—
,, 3	+ + +	+ + +	+ + +	+ + +			—	—	—	—	—	—	—
,, 4	+ + +	+ + +	+ + +	+ + +			—	—	—	—	—	—	—
,, 5	+ + +	+ + +	+ + +	+ + +									
,, 6	+ + +	+ + +	+ + +	+ + +									

+ + + = Large clumps　　　　+ = Clumps just visible to naked eye　　　　— = No agglutination

of an anti-human globulin serum. This effect is quite independent of the ABO and Rhesus group of the erythrocytes concerned. To date no human red cell has been found which cannot be fully " coated " or sensitized by exposure to the action of these eluates.

There was no appreciable difference in the degree of sensitization of erythrocytes from the different blood groups by any given eluate. It is concluded, therefore, that the cell receptor involved has no relation to any of the known blood group receptors. It is of interest to note that the erythrocytes from two cases of familial acholuric jaundice were rendered fully agglutinable in anti-human globulin serum by exposure to the eluates from Cases 1 to 4.

Some of the eluates were tested against a range of animal erythrocytes. In no case were any of the erythrocytes sensitized, with the exception of two samples of Rhesus monkey cells ; in this instance the agglutination in anti-human globulin serum after exposure to the eluate was very much weaker that in the case of human erythrocytes.

The results are tabulated in Table II.

Titre of the eluates.—The titre of the different eluates was determined using the erythrocytes of the same Group O donor. The results are shown in Table III. In addition the nitrogen content of three of the eluates, obtained by the micro-Kjeldahl procedure, is recorded in mg. N_2 per 100 ml.

TABLE III

TITRES OF ELUATES AGAINST GROUP O ERYTHROCYTES
FROM THE SAME DONOR

Eluate	Range of titres obtained with different preparations	Nitrogen content mg. per 100 ml.
Case 1	1/32 to 1/256	21.0
,, 2	Not tested	
,, 3	1/32 to 1/128	
,, 4	1/4 to 1/16	
,, 5	1/64 to 1/128	19.6
,, 6	1/16 to 1/64	32.0

Thermal amplitude.—The eluates proved to have a wide thermal amplitude. Sensitization after holding the eluate and erythrocyte mixture at 40° C. room temperature, and 37° C., seemed equally strong as judged by the degree of agglutination on subsequently suspending the washed erythrocytes in anti-human globulin serum.

Between the limits of 40° C. and 37° C., therefore, union of antibody and erythrocytes seems little affected by the temperature at which sensitization is allowed to take place.

Effect of the eluates on the osmotic fragility of normal erythrocytes.—None of the cases studied showed any marked increase of osmotic fragility in hypotonic saline. The two strongest eluates from Case 1 and Case 3 were therefore selected for use in these experiments. The experimental procedure followed is illustrated by the following experiment using eluate from Case 1. 0.25 ml. washed, packed, normal Group O erythrocytes plus 0.75 ml. eluate were incubated for one hour at 37° C. After washing three times with physiological saline and packing by centrifugation, 0.1 ml. saline was added to the deposit. Strong sensitization of the erythrocytes was confirmed at this stage by the finding of rapid agglutination of the erythrocytes on testing with anti-human globulin serum. A control of 0.25 ml. packed erythrocytes plus 0.75 ml. physiological saline was taken through the same manipulations. After thorough mixing 0.02 ml. aliquots of the two cell mixtures were distributed into a range of tubes containing 5 ml. quantities of hypotonic saline ranging from 0.3 to 0.7 g. per 100 ml. in steps of 0.05 g. per 100 ml., and a tube for complete haemolysis containing 5 ml. of 0.4 per cent ammonia. After standing for two hours at room temperature all tubes were centrifuged for ten minutes at 2,500 r.p.m. and the optical density of the supernatant was then measured in a photo-electric colorimeter using an Ilford 604 filter. The percentage haemolysis in each tube was then calculated. As a result of a number of similar experiments it was found that exposure of normal erythrocytes to the eluates from Case 1 and Case 3, even when the erythrocytes were sensitized in the presence of the patient's fresh serum, did not significantly alter their resistance to hypotonic saline.

Action of the eluates in the presence of complement.—It was obviously of interest to determine whether normal erythrocytes fully sensitized by these eluates would be haemolysed on addition of complement. It was soon found that the amount of citrate present in the eluates was sufficient to inactivate 6 to 8 M.H.D. of complement. The tests were therefore conducted by allowing the erythrocytes to react with the eluates for one hour in the 37° C. waterbath, after which they were washed three times with saline to remove all traces of buffer, and resuspended to 2.5 per cent concentration. To 0.25 ml. of the sensitized red cell suspension 0.5 ml. physiological saline and 0.25 ml. of complement dilutions containing 3 and 6 M.H.D. were then added, and the tubes kept at 37° C. for two hours and then at 4° C. for eighteen hours overnight. On removal from the ice chest the

tubes were shaken, lightly centrifuged, and examined for the presence of haemolysis. Two controls were always included:

1. 0.25 ml. sensitized cells + 0.5 ml. saline + 0.25 ml. 6 M.H.D. complement dilution previously inactivated for 30 minutes at 56° C.

2. 0.25 ml. unsensitized cells + 0.5 ml. saline + 0.25 ml. 6 M.H.D. active complement.

In Cases 1 and 2 and Cases 5 and 6 the results were unequivocal. No haemolysis was ever produced under the conditions of the experiment. In Cases 3 and 4, however, the eluates proved capable of causing haemolysis in the presence of complement. Qualitative experiments showed nearly complete haemolysis with 6 M.H.D. and partial haemolysis with 3 M.H.D. of complement. Quantitative estimation of the haemoglobin in the supernatant fluid in the two experiments showed with the eluate from Case 3 94 per cent haemolysis with 6 M.H.D. and 73 per cent haemolysis with 3 M.H.D. of complement. However, in this case the control tube containing sensitized cells plus inactivated complement also showed 20 per cent haemolysis, although the tube containing unsensitized cells plus 6 M.H.D. active complement showed no haemolysis.

Similarly in Case 4 6 M.H.D. complement gave 52 per cent haemolysis and 3 M.H.D. 19 per cent, whereas the control with inactivated complement plus sensitized cells showed 5 per cent haemolysis. The control with unsensitized cells plus 6 M.H.D. active complement again showed no haemolysis. Thus the haemolysis produced in these experiments could not have been entirely dependent on the complement activity of the guinea-pig serum, although obviously increasing the dose of complement increased the total haemolysis produced. The complete absence of haemolysis with 6 M.H.D. of complement and unsensitized cells showed that the effect could not have been due to any antihuman red cell heterolysin present in the dilutions of guinea-pig serum used.

The eluates used in both these experiments had been stored in sealed screw-capped bottles over dry carbon dioxide ice for six months. Some haemolytic activity developing on storage and not related to the antibody content of the eluate obviously cannot be excluded. Unfortunately by the time these experiments were completed neither of the patients was available for further study so that these findings could not be confirmed with fresh eluates. On the suggestion of Dr. J. V. Dacie erythrocytes fully sensitized with eluates from Case 1 and Cases 5 and 6 were suspended in

fresh human serum acidified with graded doses of HCl (Dacie and Richardson, 1943).

0.5 ml. quantities of serum were acidified with 0.05 ml. of N/10, N/5, N/4, N/3.5, N/3, and N/2.5 HCl respectively. To these aliquots of acidified serum was then added 0.05 ml. of 50 per cent washed Group O erythrocyte suspension previously fully sensitized with the eluate under test. After two hours at 37° C. the tubes were lightly centrifuged and the supernatant serum examined for the presence of haemolysis. No haemolysis was found under these conditions with any of the eluates used.

Blocking experiments.—In order to test the hypothesis that the antibody present in these eluates might be the "incomplete" form of the cold agglutinin present in the serum of some cases of acquired haemolytic anaemia the following experiment was carried out.

Two per cent washed Group O erythrocytes were sensitized with sufficient eluate to render the cells fully saturated with antibody as judged by immediate maximal agglutination on suspending a sample of the washed erythrocytes in anti-human globulin serum and the demonstration of residual antibody in the supernatant eluate. Three lots of erythrocytes sensitized with eluates from Cases 1, 5, and 6, and the same unsensitized erythrocytes, were then made up to 2 per cent suspension and used in the titration of a serum from a case of chronic haemolytic anaemia known to contain a high-titre cold agglutinin. After addition of the erythrocyte suspensions to a range of serial serum dilutions the tubes were placed in the ice chest at 4° C. for 18 hours. On removal from the ice chest the tubes were lightly shaken and the results read macroscopically and microscopically. The end point was taken as the last tube showing definite microscopic agglutination. The final titres obtained with the four lots of erythrocyte suspensions are shown in Table IV. The final titres obtained differ by only one tube and there is therefore no evidence that the action of the cold

TABLE IV

TITRE OF A COLD AGGLUTININ AGAINST UNSENSITIZED AND SENSITIZED ERYTHROCYTES AT 4° C.

Cell suspension	Titre of cold agglutinin
a. Unsensitized	1/32,000
b. Sensitized with eluate from:	
Case 1	1/16,000
Case 5	1/32,000
Case 6	1/16,000

agglutinin on the erythrocyte was blocked by previously "coating" the erythrocyte with anti-body of the type present in these eluates.

Discussion

Since the present work was begun two papers have appeared in which the elution of antibody in acquired haemolytic anaemia is described. Evans and others (1946) adduced evidence that the erythrocytes in two cases of acquired haemolytic anaemia were coated with an immune body which could be released and transferred to normal cells *in vitro*. Eluates were prepared from the washed erythrocytes of the patients by exposing them to the action of saline at 56° C. It was found that normal cells were sensitized by these eluates only if the cell-eluate mixtures were kept at 5° C. overnight and the agglutinability in anti-human globulin serum was improved by holding the cell-eluate mixtures for two hours at 37° C. after their removal from the ice chest. After five minutes at 56° C. the patients' washed cells were rendered inagglutinable in anti-human globulin serum, presumably because of elution of the adsorbed antibody. The authors further noted agglutination of the patients' cells in 30 per cent bovine albumin and 2 per cent acacia, but not in normal human serum. No serum antibodies were detected in the first patient studied. In the second patient cold agglutinin to a titre of 1/32 and an atypical agglutinin active against about 30 per cent of Group O cells irrespective of Rhesus and MN grouping was found in the serum before any blood transfusion had been given.

Sturgeon (1947) demonstrated an incomplete or blocking type of antibody in the serum of three patients with acquired haemolytic anaemia with the aid of anti-human globulin serum. The titre of free antibody ranged between 1/128 and 1/4,096 in the cases studied. In one case a high titre saline eluate was prepared by incubating the patient's cells or sensitized normal cells in equal volumes of saline for 30 minutes at 37° C. or 56° C. This was shown to react with cells of all four groups, including Rhesus-positive and Rhesus-negative cells. It did not react with Rhesus monkey cells or with sheep's cells. Cells strongly sensitized with the eluate still reacted with anti-A, anti-B, and anti-Rh sera in the normal way, thus showing that these receptors were not blocked by the new antibody. Normal cells stored for a few days in saline could not be sensitized by the eluate. The antibody was not reduced in potency by exposure to 70° C. for ten minutes, but could not be demonstrated by the indirect developing test after ten minutes at 80° C. The antibody appeared to be an auto-antibody and a pan-antibody, but did not react with Rhesus monkey cells.

The present work confirms many of these findings but there are a number of disagreements in detail. In all the cases studied it was possible by means of the acid buffer elution technique now described to elute an immune body from the erythrocytes which was capable of sensitizing or "coating" normal erythrocytes irrespective of their ABO or Rhesus groups and thus rendering them agglutinable, after washing with saline, in an anti-human globulin serum. Between the limits of 4° C. and 37° C., union of antibody and erythrocyte was not affected by the temperature at which the eluate and erythrocyte mixture was held before washing and addition of anti-human globulin serum. The eluates did not sensitize the erythrocytes of laboratory animals, with the exception of Rhesus monkey cells tested in two instances.

Saturation of normal Group O erythrocytes with the antibody contained in the two highest titre eluates did not alter their osmotic resistance to hypotonic saline. The reaction of normal Group O erythrocytes fully sensitized by eluates from Cases 1, 2, 5, and 6 in the presence of fresh guinea-pig serum and fresh human serum suitably acidified was clear-cut. No haemolysis was ever found under these conditions. Using the eluates from Cases 3 and 4, however, haemolysis was consistently produced in the presence of guinea-pig complement, but the experiments were complicated by the finding of some degree of haemolysis in the presence of a 6 M.H.D. complement dilution inactivated by heating for thirty minutes at 56° C. The complete absence of haemolysis of unsensitized cells suspended in the same dilution of active complement was held to exclude the presence of any anti-human heterolysin in the dilutions of guinea-pig serum used. The possibility that this effect was related to prolonged storage of the eluates in the frozen state is considered.

A limited experiment showed that saturation of normal erythrocytes with the antibody contained in the eluate from three of the cases did not block the action on the erythrocytes of a high-titre cold agglutinin present in the serum of a patient with chronic haemolytic anaemia.

The experiments reported show that the immune body present in these eluates reacts with a broad antigen present in all human red cells and to a lesser extent in Rhesus monkey cells. Although direct evidence cannot be produced on this point it seems likely that the cell receptor involved is closely related to the human species-specific receptor reacted upon by anti-human red cell sera pre-

pared in other species. The general behaviour of the antibody, apart from its broader specificity, shows a close resemblance to that of the " incomplete " anti-Rh antibody and especially to that form designated " cryptagglutinoid " by Hill and others. (1948).

In contrast to the findings of Sturgeon (1947) and Neber and Dameshek (1947) using 30 per cent bovine albumin no free antibody of " incomplete " type could be demonstrated in the serum of any of the present cases using the anti-human globulin serum technique.

The *in vitro* reactions, as in the case of the " incomplete " anti-Rh antibody, shed little light on the mechanisms by which haemolysis is produced *in vivo*. Much further work is required on this question, and it is hoped that the elution technique outlined may help in such investigations.

Summary

1. A technique is outlined whereby an antibody of " incomplete " type can be eluted from the erythrocytes in acquired haemolytic anaemia.

2. The properties and *in vitro* reactions of such eluates are described and discussed with reference to previously published findings.

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SURVIVAL AFTER TRANSFUSION OF Rh-POSITIVE ERYTHROCYTES PREVIOUSLY INCUBATED WITH Rh ANTIBODY

BY

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It has been concluded that if blood is taken from a patient with acquired haemolytic anaemia and transfused to a normal recipient the erythrocytes survive normally (Loutit and Mollison, 1946). This finding is not an easy one to understand since the erythrocytes at the moment of transfusion are coated with antibody and give a positive direct Coombs test, and it might be thought that if the degree of sensitization were adequate only the addition of complement was necessary to complete their lysis. In discussing this finding it was suggested that immune body might be washed off the erythrocytes when they were put into the circulation of a normal patient. It was further suggested that there was probably some other substance, " co-haemolysin," present in the plasma of patients with acquired haemolytic anaemia in addition to the antibody giving the positive Coombs reaction. Some such theory was necessary to explain the findings in the case of a patient who recovered spontaneously from acquired haemolytic anaemia and no longer destroyed transfused erythrocytes rapidly although her own red cells continued to give a positive direct Coombs test.

Owren (1947) made some observations which were a little at variance with those reported above. He took erythrocytes from a patient with acquired haemolytic anaemia and transfused them to a normal recipient. Approximately 50 per cent of the erythrocytes disappeared from the recipient's circulation within three days, and during this period it could be demonstrated that the surviving donor cells gave a positive direct Coombs test. After three days the surviving cells no longer gave a

positive test and were eliminated at the normal rate only. This observation suggested that the coating of erythrocytes with antibody did in fact lead to their rapid destruction, and that the idea that antibody could simply be washed off the erythrocytes, leaving them undamaged, might be wrong.

It seemed desirable to find out first whether erythrocytes coated with antibody, or " sensitized erythrocytes " as they may conveniently be called, lose their coat of antibody when circulating in the plasma of a normal recipient, or whether they retain it; and secondly to make further observations on the effect of this coating on the survival of erythrocytes.

A further incentive to perform experiments came from the observation that an infant might have a positive direct Coombs test without developing any signs of increased blood destruction (Pickles, 1947; Mollison and Cutbush, 1949).

Methods

Donor Blood.—Concentrated erythrocyte suspensions prepared from blood stored for three to four days in disodium-citrate glucose were used. Varying amounts of serum containing Rh antibody of the " albumin agglutinin " or " blocking " type were added (see the Table, and note that in one case a serum containing saline agglutinins was also added). The mixtures were incubated in a water-bath at 37° C. for one hour. A sample of the mixture was then withdrawn and the direct Coombs test was performed. The mixture was then transfused to a patient. In one preliminary experiment (Case 1) blood was mixed with a very small amount of weak Rh antibody. Since

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TABLE

Case no.	Name	Sex	Age (yrs.)	Clinical condition	Donor blood			Direct Coombs test after incubation	Persistence of positive direct Coombs test on patient's blood after transfusion (days)
					Volume of packed cells (ml.)	Anti-Rh serum			
						Volume (ml.)	Titre		
1	Ch.	F	6/12	Microcytic anaemia	50	2	64	+ (weak)	1
2	Er.	F	51	Anaemia following haemorrhage at operation	450	80	16	+ +	4
3	Bo.	M	59	do.	260	{ 120 150	256 32*	} + + + +	58
4	Lu.	F	56	Rheumatoid arthritis	420	120	128	+ + +	32
5	Co.	M	64	do.	480	60	64	+	not done

* This anti-Rh serum contained saline agglutinins; all the other sera used were of the albumin-agglutinin or "blocking" type.

there were no signs at all of increased blood destruction when this mixture was transfused, larger proportions of anti-Rh serum were used in the succeeding cases.

Recipients.—Survival tests were carried out on five patients, all of whom required transfusion for the relief of chronic anaemia. Brief details are given in the Table. All the recipients were Rh-positive.

Survival of transfused erythrocytes was determined by a modification (Dacie and Mollison, 1943) of Ashby's (1919) method.

Direct Coombs Test.—The test described by Coombs, Mourant, and Race (1945) was used ; the precise technique used in these experiments was that described by Mollison and Cutbush (1949).

Results

Survival of Sensitized Erythrocytes.—The survival of sensitized erythrocytes in two patients (Cases 2 and 3) is plotted in Fig. 1. The percentage survival at ten days in both cases is a little lower than normal, although the remainder of the curve is strictly normal. The apparent initial departure from linearity in these cases is too small to be taken as definite evidence of an increased rate of destruction. Nevertheless it is a suggestive finding in view of similar small deviations from normality in the cases of Loutit and Mollison. The question is discussed below.

The survival of sensitized erythrocytes in two patients with rheumatoid arthritis (Cases 4 and 5) was appreciably shorter, 50 per cent of transfused erythrocytes being eliminated in about 22 days. In view of the results in Cases 2 and 3 it was felt that this reduced survival might be due to some abnormality of the recipient rather than to any effect of Rh antibody on the donor erythro-
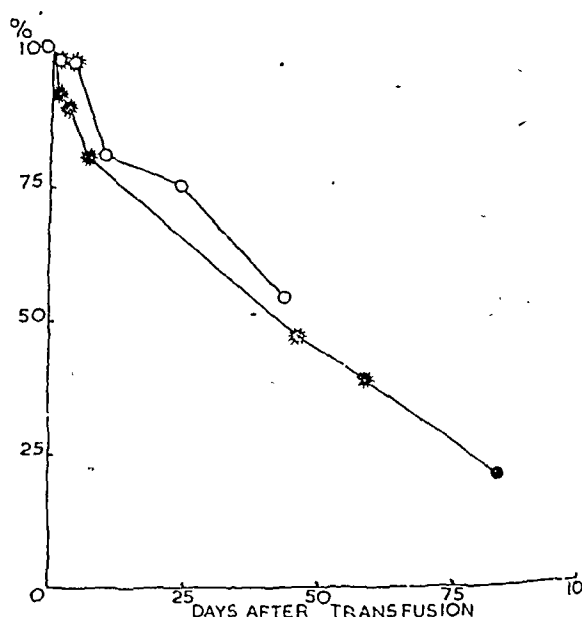
FIG. 1.—Survival of " sensitized " cells in Case 2 (open circles) and Case 3 (solid circles). Starred circles show direct Coombs test positive.

cytes. This supposition was tested by retransfusing the two recipients at a later date with normal blood. In Case 4 elimination of the normal erythrocytes was just as rapid as that of the sensitized erythrocytes (see Fig. 2). In Case 5 normal erythrocytes survived normally. However, in this patient there had been some clinical improvement since the time when the transfusion of sensitized erythrocytes had been given. In view of the results in the other patient with rheumatoid arthritis, it is possible that normal survival on the second occa-

sion is rather to be ascribed to an alteration in the disease process than to any difference between normal and sensitized erythrocytes, especially since the sensitized cells transfused to this patient were only weakly sensitized.
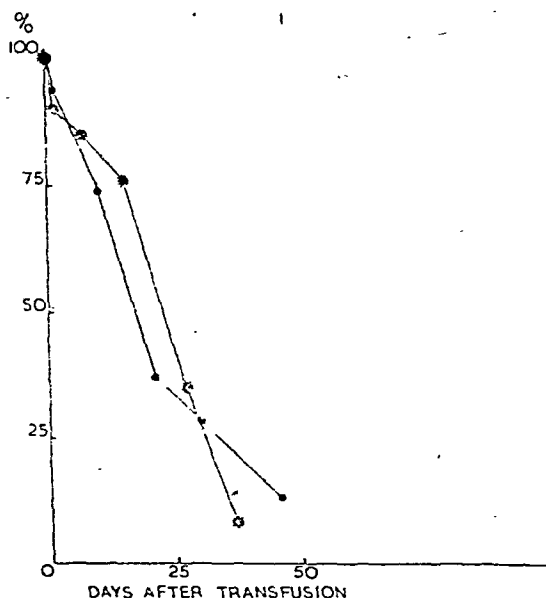
FIG. 2.—Survival in Case 4 of "sensitized" and normal cells transfused on different occasions. Starred circles show direct Coombs test positive (after transfusion of sensitized cells).

Persistence of a Positive Direct Coombs Test.— In Cases 1, 2, 3, and 4 a direct Coombs test was performed on the recipient's blood at intervals after transfusion. In Case 1, in which the donor cells were only weakly sensitized, the direct Coombs test was positive on the day after transfusion but not thereafter ; in Case 2, in which the donor cells were moderately sensitized before transfusion, the direct Coombs test was positive four days after transfusion but negative at ten days. However, in Case 3, in which the donor cells were very strongly sensitized, the direct Coombs test was still positive 58 days after transfusion. In Case 4 the direct Coombs test was positive for 32 days after transfusion.

In cases where the recipient's blood became " Coombs positive " after transfusion, it was noted that all the erythrocytes, and not just a fraction, corresponding to the proportion of donor cells present, were agglutinated.

This general sensitization of the recipient's erythrocytes was not accompanied by any signs of an increased rate of blood destruction. In the

patient (Case 3) in whom the direct Coombs test remained positive for 58 days after transfusion, some special examinations were made on the 46th day after transfusion. The haemoglobin was 9.5 g. compared with 9.6 g. immediately after transfusion, the reticulocyte count was 0.6 per cent, and the plasma bilirubin concentration less than 0.5 mg./100 ml.

A few simple experiments were undertaken to discover how rapidly Rh antibody was transferred from sensitized Rh-positive cells to unsensitized ones.

Experiments in Vitro

I. *Sensitized Rh-positive Cells Incubated with Rh-positive and Rh-negative Cells Respectively*

Suspensions of erythrocytes in saline were prepared from (a) the blood of an infant with haemolytic disease, (b) normal Rh-positive blood, (c) normal Rh-negative blood.

In each case the erythrocytes were washed three times in saline and then made up to a suspension of approximately 50 per cent in saline. Mixtures of these suspensions were made as below and incubated for one hour at 37° C.

(1) 0.4 ml. *a* alone.
(2) 0.5 ml. *a*+0.5 ml. *b*.
(3) 0.5 ml. *a*+0.5 ml. *c*.

After incubation samples of (1), (2), and (3) were each tested against an anti-globulin serum. The results were as follows:

(1) The sensitized cells alone showed a very strong positive reaction.

(2) The mixture of sensitized cells with the normal Rh-positive cells showed slower reaction, but agglutination was complete eventually.

(3) The mixture of sensitized cells with Rh-negative cells gave the appearance of a mixture of well agglutinated and unagglutinated cells.

To confirm the impression gained from these macroscopic tests, microscopic tests were done as follows.

Small volumes of (1), (2), and (3) were mixed with equal volumes of anti-globulin serum in tubes and centrifuged. Then 10 ml. saline were added to each tube and mixed gently ; the mixtures were spun once more and again gently mixed. A drop of each mixture was now examined in a counting chamber.

With the sensitized cells alone (1) there were only three to five free cells per broad column of the Bürker ruling (i.e., agglutination was virtually complete). With the mixture of sensitized and normal Rh-positive cells (2) there were only ten cells per broad column (i.e., agglutination virtually complete, but not quite so strong). With the mixture of sensitized cells and Rh-negative cells (3) there were very numerous free cells and many small clumps. From this experiment it was concluded that all the normal Rh-positive cells had become coated with antibody during incubation with sensitized Rh-positive cells.

II. *Sensitized Rh-positive Cells Incubated in Excess Normal Plasma*

1 ml. of normal Rh-positive blood was incubated with 0.1 ml. serum containing Rh antibody (albumin titre 128, saline titre nil); the cells were then washed and made up to a 20 per cent suspension in saline. 0.1 ml. of these sensitized cells was incubated for one hour with 2 ml. normal plasma. At the end of this time the cells were tested in parallel with sensitized cells that had not been mixed with excess plasma to discover whether any appreciable amount of antibody had come off the cells during incubation. This latter possibility was also tested by performing an indirect Coombs test on the plasma after incubation.

It was found that there was no detectable diminution in the strength of the direct Coombs test after incubating the cells in excess plasma and that no free antibody was detected in the plasma. From this it was concluded that Rh antibody does not readily wash off red cells to which it has become attached, unless other Rh-positive erythrocytes are present.

Discussion

If the results of Loutit and Mollison (1946) are examined closely two things will be noted. Firstly, with regard to the patients with acquired haemolytic anaemia from whom blood was taken for transfusion to normal recipients, it will be observed that in none of the four cases was haemolysis more than moderately active at the time of the experiment. Thus one patient had made a clinical recovery, two patients (previously severely affected) had had their spleens removed, and the fourth case was mild. Nevertheless in two cases (those in which blood destruction had been very rapid before splenectomy), there was in fact some evidence that a small proportion of the cells was destroyed more rapidly than normal after transfusion to normal recipients; the exact figures were 79 per cent and 84 per cent survival respectively one week after transfusion, compared with an expected figure of about 93 per cent. Loutit and Mollison drew attention to this finding, but laid more stress on the normal survival of the main mass of the transfused cells.

In the present two patients (Cases 2 and 3) in whom the bulk of the transfused erythrocytes also survived normally, the percentage survivals, seven to ten days after transfusion, were 81 per cent and 82 per cent respectively. Thus it seems quite likely that, although the bulk of the sensitized erythrocytes survived normally, there may have been a fraction which was destroyed more rapidly. If this is so, the substantial initial destruction observed by Owren may have been merely an exaggeration of the same effect. Two factors in his case may have contributed to this result. Firstly, it may be

that the haemolytic process in his patient was far more active at the time of transfusion than in the cases of Loutit and Mollison. Secondly, Owren transfused a larger volume of blood—namely, 1½ litres. The effect which this may have had demands separate consideration.

The tests *in vitro* demonstrate that Rh antibody is readily transferred from sensitized Rh-positive cells to unsensitized Rh-positive cells. This implies that within a very short period of transfusion, probably less than an hour, the amount of antibody originally attached to the donor erythrocytes is shared among all the circulating erythrocytes. If only some 500 ml. of blood are transfused, this must reduce the amount of antibody on each cell to about 1/6th or less of the amount initially attached to the donor cells, even when the recipient has a reduced erythrocyte volume. In Owren's case, due to the larger amount of sensitized blood transfused, the reduction in the amount of antibody on each of the donor erythrocytes must have been less.

It may have been a coincidence that in Owren's case the direct Coombs test, performed on the recipient's cells, became negative at the same time as the rapid destruction ceased. The time for which the test remains positive depends to a large extent on the sensitivity of the Coombs' reagent. In any case, our observations demonstrate that the direct Coombs test can remain positive over quite long periods without leading to any increase in the rate of erythrocyte destruction.

Summary

1. Rh-positive erythrocytes were incubated *in vitro* with serum containing Rh antibody of the albumin-agglutinin type, and then transfused to anaemic patients. The erythrocytes were found to retain their coat of antibody, as demonstrated by the direct Coombs test, for long periods after transfusion.

2. When Rh-positive erythrocytes, sensitized *in vitro*, are transfused to an Rh-positive recipient, the Rh antibody becomes redistributed amongst the erythrocytes of both the donor and recipient. Hence the amount of antibody attached to the donor erythrocytes must be rapidly reduced.

3. Whereas the bulk of the sensitized erythrocytes survive normally after transfusion, it is possible that a small proportion may be destroyed more rapidly. Further experiments are needed to decide this point.

4. These findings can be interpreted in two ways. (*a*) The coating of erythrocytes by Rh antibody

determines the haemolysis of erythrocytes *in vivo* only when a certain minimal quantity of antibody is exceeded ; (*b*) the antibody detected by the anti-globulin test does not by itself, or in co-operation with any normal plasma constituent, cause haemolysis.

5. Whatever the interpretation of these findings, it is clear that the finding that erythrocytes give a positive direct Coombs test cannot by itself be taken as evidence that the erythrocytes are being destroyed at an abnormal rate.

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ACQUIRED HAEMOLYTIC ANAEMIA: SURVIVAL OF TRANSFUSED ERYTHROCYTES IN PATIENTS AND NORMAL RECIPIENTS

BY

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Evidence of a difference in the mechanism of haemolysis between congenital and acquired haemolytic anaemia (acholuric jaundice) has been revealed by studies of the survival of transfused erythrocytes and by the direct Coombs test (Coombs and others, 1945a, b). Dacie and Mollison (1943) showed that normal erythrocytes transfused into cases of congenital haemolytic anaemia survived normally, but the erythrocytes from such cases when transfused into normal persons were rapidly eliminated. Moreover, these erythrocytes give a negative direct Coombs test (Boorman and others, 1946) and are referred to in this paper as "unsensitized." In contrast, erythrocytes from cases of acquired haemolytic anaemia give a positive direct Coombs test (Boorman and others, 1946) and may thus be referred to as "sensitized." Normal erythrocytes transfused to these patients are rapidly eliminated, but the patients' own erythrocytes survive normally when transfused into normal persons (Loutit and Mollison, 1946). This evidence, Loutit and Mollison suggested, indicates that the erythrocytes in acquired haemolytic anaemia are sensitized by an antibody or haemolysin; and their normal survival when transfused into a normal circulation may be explained by supposing either that the sensitization is reversible in the medium of a normal circulation, or that in acquired haemolytic anaemia there is an additional factor causing the rapid elimination of the sensitized cells.

We have attempted to determine which of these suppositions is correct, and to ascertain whether transfused normal cells become sensitized before elimination from the circulation of patients with acquired haemolytic anaemia. We have examined the erythrocytes from five patients, all of whom gave a positive direct Coombs test, and we present in this paper the detailed results of our investigation of three of these patients. The fate of normal erythrocytes transfused to these patients and of the erythrocytes from one of them (Case 2) transfused to two normal recipients was studied by means of differential agglutination counts employing Mollison's (1947) modification of Ashby's (1919) method. This technique was further modified to enable sensitized and unsensitized cells to be distinguished from each other: that is, "differential direct Coombs counts" were undertaken.

Experimental Methods

The transfused erythrocytes in the recipient's circulation were estimated by differential agglutination tests as a percentage of the total erythrocyte count. An approximately 2 per cent cell suspension in isotonic (0.85 per cent) saline was made from the recipient's blood. This suspension was mixed in known volumes in glass tubes ($2\frac{1}{2}$ in. by $\frac{1}{2}$ in.) with isotonic saline and with the chosen anti-serum to give in each case a final cell suspension of approximately 0.1 per cent. The total number of cells per c.mm. in the saline mixture was counted. The anti-serum cell mixture was treated as follows (Mollison, 1947):

(1) The mixture was allowed to stand for at least 1 hour at room temperature, or at 37° C. if anti-Rh serum was being used; (2) it was centrifuged at 1,000 r.p.m. for 1 minute, and the deposit resuspended by shaking with moderate vigour; (3) centrifuging and resuspension were twice repeated; (4) the tube was stood vertically for a few seconds to allow the larger clumps to sediment, and a drop of fluid was transferred from the top of the column to each of two counting-chambers, using a Pasteur pipette; (5) the unagglutinated cells were counted, and from the average count per c.mm. the percentage of unagglutinated cells in original suspension was calculated; the

number per c.mm. in the recipient's blood was then derived from the total erythrocyte count.

Differential direct Coombs counts were performed in a similar manner. Suspensions of thrice-washed cells in isotonic saline were treated as described above after having been mixed with volumes of fully absorbed high titre anti-human-serum rabbit serum prepared at this centre by the method of Coombs and Mourant (1947). This serum was used at a final dilution of 1/100 (full titre against cells sensitized with anti-Rh incomplete antibody being 2,000), and the mixture was allowed to stand for half an hour.

The accuracy of the differential Coombs count was tested with known mixtures of normal cells and cells sensitized *in vitro* with an incomplete anti-D serum, and with mixtures of normal cells and sensitized cells from one of the patients described below. The results always agreed with the calculated percentage of sensitized cells within the limits of ±5 per cent. With pure suspensions of sensitized cells the unagglutinated cells numbered about 1 per cent of the total.

The number of sensitized cells per c.mm. of the recipient's blood was calculated as above. The recipient's own erythrocyte count was derived from the total and transfused erythrocyte counts. Reticulocytes were estimated as a percentage from a stained blood film and the actual count per c.mm. calculated.

FIG. 1.—Progress of Case 1.

Case Reports

A. THE FATE OF NORMAL ERYTHROCYTES TRANSFUSED* TO PATIENTS WITH ACQUIRED HAEMOLYTIC ANAEMIA

Case 1.—A man aged 70 years had had symptoms for two years. He had moderate pallor and icterus and an enlarged spleen. The blood film showed moderate anisocytosis and polychromasia, 50 per cent reticulocytes, and very occasional late normoblasts. There was raised serum bilirubin and urinary urobilin, and markedly increased erythrocyte fragility with spherocytosis. No cold auto-agglutinins, immune agglutinins, or incomplete antibodies were demonstrable in serum. The direct Coombs test on erythrocytes was strongly positive. The blood group was B, D-positive. Normal group B, D-negative blood was transfused, and differential counts were carried out with a specific anti-D agglutinating serum (titre 512 and used at a final dilution of ¼) from a group AB, D-negative woman immunized by pregnancies.

Two days after a transfusion of 2 pints of packed cells (Fig. 1) the circulating blood contained 900,000 sensitized cells per c.mm. of blood and 250,000 of the patient's own cells per c.mm. Four days later the total of sensitized cells had increased by 780,000 per c.mm., although the patient's own cells had increased by only 350,000 per c.mm. From these findings it was concluded that some of the transfused cells had become sensitized during the latter four days.

From the sixth to the twelfth day the sensitized cell count fell steadily and rapidly, parallel to the fall in the transfused cell count; the patient's own cell count

*Stored blood was used for the transfusions in this group of patients. The percentage of non-viable cells it contained in each case was calculated from the results of Ross and others (1947): it was approximately 5 per cent, 3 per cent, and 2 per cent respectively in Cases 1, 2, and 3. Most of these non-viable cells would have been eliminated in two hours after entering the circulation of the recipient, and the remainder within 24 hours.

The blood for transfusion was cross-matched by incubating a cell suspension in saline with the recipient's serum at 37° C. for at least 1 hour. No agglutination was observed to have occurred and an indirect Coombs test was then performed on the red cells, which were negative even when the incubation was prolonged on one occasion (Case 1) for 6 hours, and on another (Case 2) for 24 hours.

slowly rose. These changes support the conclusion that a proportion of the sensitized cells were transfused cells.

Case 2.—A woman aged 42 years had had symptoms for three months. She had marked pallor with slight icterus and an enlarged spleen. The blood film showed moderate anisocytosis and polychromasia, 20 per cent reticulocytes, and very occasional late normoblasts. The serum bilirubin and urinary urobilin were raised, and the erythrocyte fragility was slightly increased. The patient's serum contained a cold auto-agglutinin (in saline dilutions titre 4 at 4° C., 1 at 15° C., no activity at 37° C.), but no immune agglutinins or incomplete antibodies were demonstrable. The direct Coombs test was strongly positive; differential tests showed that 96 to 99 per cent of the erythrocytes were sensitized. The blood group was O, D-negative type MN. Normal group O, D-negative, type N blood was transfused, and a specific naturally-occurring anti-M serum (titre 32) from a blood donor was used at a final dilution of ¼ in the differential agglutination counts. (This anti-M serum did not cause any non-specific agglutination such as may be found with artificially prepared anti-M or anti-N rabbit sera.)

This patient destroyed transfused cells very rapidly: on one occasion the transfused cell count fell from 1,550,000 per c.mm. to 500,000 per c.mm. of blood in 7 days, an elimination of 63 per cent.

In Fig. 2 the M-negative cells present at the start were survivors of previous transfusions. Immediately after the transfusion of 1½ pints of packed cells there were 1,300,000 M-negative cells per c.mm. of blood. However, the unsensitized cell count had risen by only 380,000 per c.mm., which suggested that the majority of transfused cells had already become sensitized. Eighteen hours after the end of the transfusion all the cells were sensitized, the increase in the transfused cell count being probably due to the adjustment of circulating blood volume after a blood transfusion (Mollison, 1947). From this time onwards the transfused cell count decreased rapidly—59 per cent of the cells being eliminated in forty-eight hours.

Case 3.—A girl aged 10 years had pallor and slight enlargement of liver and spleen. Many reticulocytes could be seen in the blood film, and the bone marrow was normoblastic. The serum bilirubin and urinary urobilin were raised, and the erythrocyte fragility was at the upper limit of normal. The direct Coombs test was strongly positive, 96 per cent of the cells being sensitized. Her serum contained a cold auto-agglutinin at a similar titre to that of Case 2; no immune agglutinins or incomplete antibodies were demonstrable. The blood group was A, D-positive. Blood of group O, D-positive, was transfused, and a stock grouping anti-A serum (titre 512) was used at a final dilution of ¼ for the differential agglutination counts. Fig. 3 shows the results obtained after the anaemia had already been relieved to a certain extent by group A blood transfusions.

Ten hours after the transfusion of 2 pints of blood the group O cell count had risen by 1,490,000 per c.mm. of blood, and the unsensitized cell count by 430,000 per c.mm. Presumably, approximately 1,060,000 per c.mm. of the transfused group O cells had become sensitized. Two days after the transfusion the unsensitized cell count had fallen to its base-line of about 100,000 per c.mm. of blood, indicating that all the transfused cells had become sensitized. The transfused cells were rapidly eliminated, 45 per cent within ten days.

B. THE FATE OF ERYTHROCYTES FROM PATIENTS WITH ACQUIRED HAEMOLYTIC ANAEMIA WHEN TRANSFUSED TO NORMAL RECIPIENTS

Case 4.—The second part of the investigation was carried out with

Transfusion : 710 ml. packed cells

FIG. 2.—*Progress of Case 2. Lower half shows mottling.*

blood taken from Case 2 into acid-citrate dextrose solution. The cells settled rapidly owing to the presence of the cold auto-agglutinin and, after half an hour, 80 ml. of the settled cells were transfused into a two-weeks-old baby who had inoperable spina bifida with paraplegia (Case 4). Ninety-six per cent of the cells were sensitized. The recipient's cells were Group A, and before the transfusion were completely unsensitized.

Differential agglutination counts twenty-four hours after the transfusion (Fig. 4) showed that 1,350,000 Group O cells per c.mm. of blood had been introduced by the transfusion and that there were 1,900,000 sensitized cells per c.mm. As the sensitized cell count rose even higher during the succeeding days, it became apparent that the recipient's cells were becoming sensitized as the result of the transfusion.

About 50 per cent of the transfused cells were eliminated during the next three days, but subsequently the rate of disappearance became less and less rapid until from about the sixteenth day onwards the cells were being eliminated at the rate of normal healthy adult cells. The transfused cell counts have been corrected for changes in the recipient's body-weight.

In order to determine whether the surviving transfused cells were still sensitized, as complete a separation as possible of the transfused cells was made from the recipient's blood in the following way: A heavy cell suspension of the recipient's blood in saline was made, and anti-A serum added as for a differential agglutination count. After the third centrifuging the supernatant fluid was removed and the cells resuspended in saline. After the large clumps had been allowed to settle for about a quarter of an hour the supernatant suspension was removed and subjected to the differential agglutination procedure again. After the third centrifuging the cells were resuspended and allowed to settle for half an hour. The supernatant suspension was removed, and the cells in it washed and tested with anti-human-serum rabbit serum.

The final suspension so obtained on the thirty-eighth day after transfusion contained 100 per cent Group O cells (transfused cells), of which 97 per cent were sensitized. On the forty-fifth day a similar suspension contained 88 per cent Group O cells, and again 97 per cent were sensitized. These results indicate that the transfused cells had remained sensitized whilst in the recipient's circulation.

The sensitized cell count of the recipient's blood after the transfusion rose steadily for nine days, until
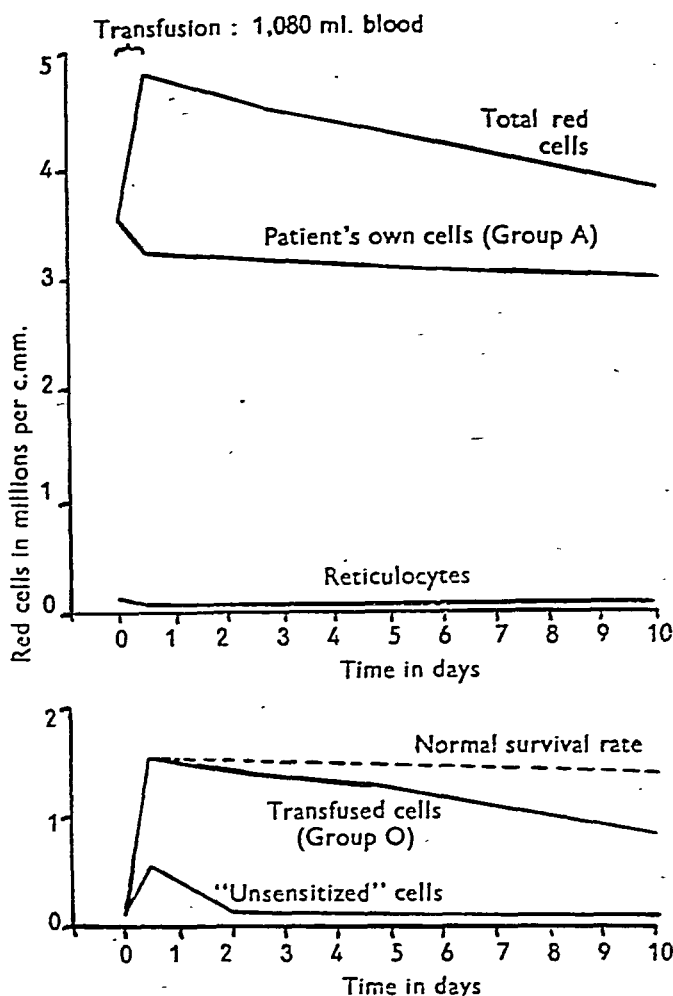
Fig. 3.—Progress of Case 3.

65 per cent of the recipient's own cells were sensitized, assuming the transfused cells to be sensitized as the above results indicate. One month later the recipient's general condition deteriorated spontaneously because of the congenital neurological abnormality, and the total erythrocyte count fell markedly. Eighty-five per cent of the recipient's own cells were then sensitized.

Case 5.—Seven weeks after Case 2 had undergone splenectomy and had improved thereby, blood was again taken from her into acid-citrate dextrose solution. After the cells had settled for one hour at 4° C., 100 ml. of the supernatant plasma were transfused into a recipient of blood Group B and similar to Case 4. No sensitized cells were detected in the recipient's blood during the following six days. Later, 100 ml. of whole blood from Case 2 were transfused, 98 per cent of the cells in this blood being sensitized.

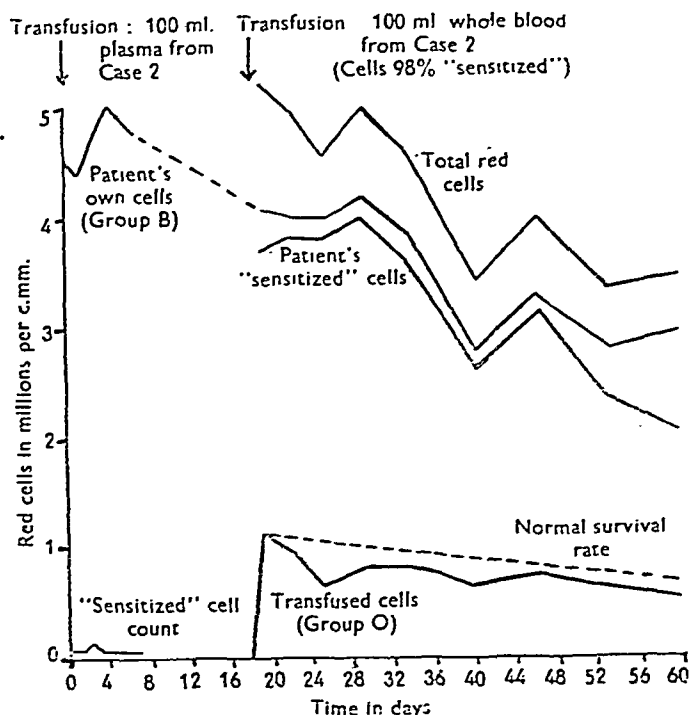Transfusion : 80 ml. packed cells from Case 21
(96% "sensitized")
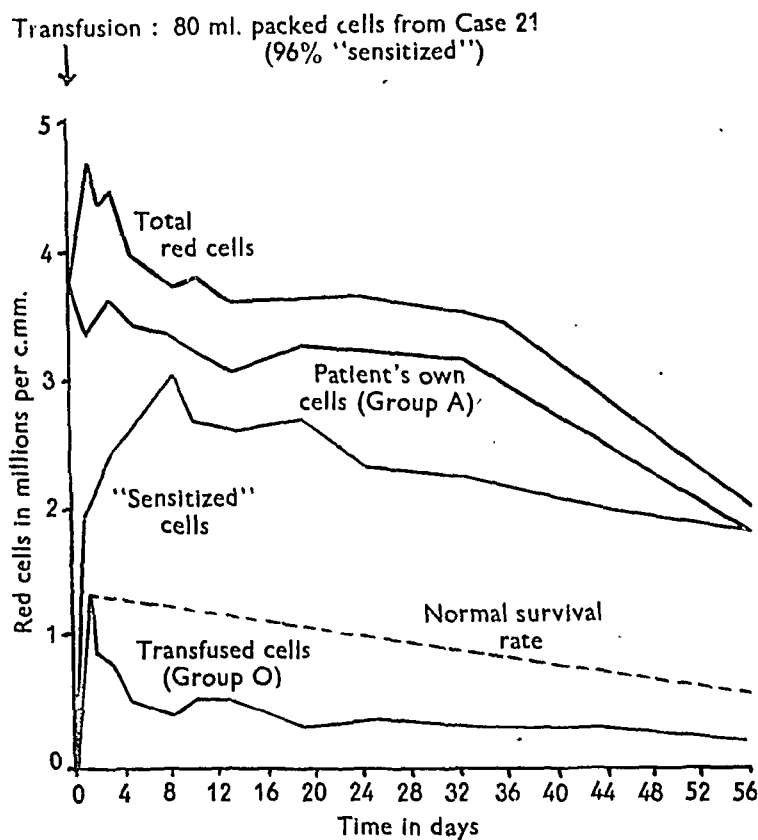
FIG. 4.—Differential agglutination counts after transfusion of Case 4 with blood taken from Case 2 into acid-citrate dextrose solution.

After twenty-four hours there were 1,150,000 Group O cells per c.mm. of blood and 4,860,000 sensitized cells per c.mm. out of a total erythrocyte count of 5,230,000 per c.mm. As shown in Fig. 5, the transfused Group O cells have been assumed to be still sensitized and for purposes of charts their total has been subtracted from the total sensitized cell count so as to give the recipient's sensitized cell count. It thus appears that about 95 per cent of the recipient's own cells become sensitized, and that this percentage remained constant during the next twenty-seven days, after which it started to decrease, falling to 70 per cent by the forty-second day after the transfusion.

To check the assumption that the transfused cells were still sensitized, a suspension of transfused cells was prepared on the twenty-eighth day after the transfusion by the method described above (Case 4). Differential counts showed it to contain 100 per cent Group O transfused cells, 90 per cent of which were sensitized.

The transfused cells were eliminated more slowly than in Case 4 : 30 per cent disappeared during the first ten days, but thereafter they were eliminated at the rate of normal adult cells. The counts have been corrected for changes in the recipient's body-weight.

Discussion

The erythrocytes of patients with acquired haemolytic anaemia give a positive direct Coombs test (Boorman and others, 1946), suggesting that a change has taken place on the cell surface, perhaps due to the adsorption of an immune auto-antibody.

Our finding that normal cells become sensitized and give a positive direct Coombs test when transfused into a patient with acquired haemolytic anaemia, before being eliminated from the recipient's circulation, provides further evidence that an antibody or sensitizing agent initiates the haemolytic process. This antibody is evidently non-specific; and following sensitization the transfused cells are eliminated more rapidly than is normal.

Our investigations have confirmed the findings of Loutit and Mollison (1946) that cells from cases of acquired haemolytic anaemia can survive normally when transfused into a normal recipient. This fact, Loutit and Mollison suggested, indicates that sensitization *per se* is not enough to ensure the premature haemolysis of cells in acquired haemolytic anaemia ; and they have postulated the presence in such cases of either a co-haemolysin or a " pathologically potentiated spleen," either of which might complete the destruction of the cells. The survival of sensitized cells in a normal circulation could then be explained by the absence of the additional haemolytic mechanism, or alternatively the sensitized cells might lose their sensitization in a normal circulation.

We have shown that such cells remain sensitized during their life in the normal circulation, and thus it seems that their increased resistance under such conditions is not due to the sensitization having been reversed.

The theory that a pathologically potentiated spleen may cause the rapid destruction of the sensitized cells is supported by the fact that an enlarged spleen is a characteristic finding in acquired haemolytic anaemia, and that splenectomy is followed in some cases by clinical improvement. Of the three patients studied by us, however, Cases 1 and 2 were treated only by

repeated blood transfusions. They have remained well without further treatment for eighteen months and four months respectively. Case 3, after similar transfusion therapy, had a clinical remission, but a month later she relapsed and splenectomy was performed. She has since remained well for three months. Therefore it seems that any abnormal splenic activity in this condition must be very variable.

Our finding that a proportion of the patient's cells are rapidly eliminated in a normal subject suggests that it may not be necessary to postulate the presence of an accessory haemolytic factor. Although the fate of the red cells may not be finally determined by the sensitization itself, it may depend on the *degree* of sensitization. Blood from two of the patients with acquired haemolytic anaemia studied by Loutit and Mollison (1946) was eliminated at a faster rate than normal for the first week after transfusion to normal subjects, but survived normally thereafter. In both our cases this initial rapid elimination rate was seen, but once again survival was subsequently normal.

FIG. 5.—Differential agglutination counts after transfusion of Case 5 with blood taken from Case 2 into acid-citrate dextrose solution.

Brown and others (1944) showed that, when the normal elimination of normal transfused cells is plotted as a graph, the result is linear, while elimination by a haemolytic mechanism results in an exponential curve. Figs. 4 and 5 show that for approximately the first ten to fifteen days a haemolytic mechanism was at work, but that after this time it ceased to act. The elimination subsequently continued in the normal linear way, in spite of the transfused cells still remaining sensitized. This could be explained by supposing that haemolysis of sensitized erythrocytes in the normal body occurs only amongst those cells which are heavily sensitized. Those which carry more than a certain threshold dose are rapidly eliminated, others less heavily sensitized surviving normally. It is reasonable to suppose that some cells from a patient with acquired haemolytic anaemia carry a heavier dose of antibody than do others. The fact that it has always been so difficult to demonstrate free antibody in the sera of such cases (Neber and Dameshek, 1947) indicates that it is taken up by the circulating cells very soon after it has been formed, and so these cells which have been circulating longest may carry more antibody than the newcomers. If this is so it would seem possible that the abnormal haemolysis in acquired haemolytic anaemia depends upon the continued sensitization of the patient's erythrocytes by a non-specific auto-antibody until sensitization reaches a certain threshold. When this threshold is reached the cells are eliminated by the normal processes of the body.

Mollison (1948) transfused infants with normal cord blood and found that the transfused cells were relatively rapidly eliminated during the first ten days and that this was followed by a normal rate of elimination. He suggested that the macrocytes in newborn infants' blood might survive less well than the normocytes. Our last two cases (4 and 5) were infants who received blood containing many macrocytes. The presence of these macrocytes may in part be responsible for the initial rapid elimination of transfused cells in these two cases.

The spread of sensitization among the normal cells of our last two cases, as shown in Figs. 4 and 5, is probably due to a redistribution of the

antibody actually attached to the transfused red cells, since no demonstrable sensitization occurred after the transfusion to Case 5 of fresh plasma from Case 2. This redistribution could be explained by supposing either that the antibody is set free again when a transfused cell is haemolysed and that it re-attaches itself to a normal cell, or that it is a simple sharing of the antibody between the transfused cells and the recipient's normal cells. Evidence for the former supposition is found in the fact that the peak in the spread of sensitization among the recipient's cells in Fig. 4 was reached at the same time as the exponential mechanism acting on the transfused cells gave way to a normal linear elimination.

Although the majority of the recipient's cells (Cases 4 and 5) became sensitized as a result of the transfusion of blood from a case of acquired haemolytic anaemia, no ill-effects, reticulocytosis, or other signs of acquired haemolytic anaemia were observed.

The Coombs test, at present, is purely qualitative and does not indicate how strongly a cell may be sensitized. It might be possible to modify the counting technique, perhaps by employing lower concentrations of anti-globulin serum, so as to show that some cells are more easily agglutinable with dilute anti-serum than others. In this way further evidence might be obtained as to the relationship between the degree of sensitization and the abnormal breakdown of the erythrocytes in acquired haemolytic anaemia.

Summary

1. The survival of normal erythrocytes transfused to three patients with acquired haemolytic anaemia was studied by means of differential agglutination counts. Differential direct Coombs counts were also performed and the results indicate that the transfused cells become " sensitized " before being eliminated at a rate greater than normal.

2. Blood from one of these patients transfused into two normal recipients was eliminated at an increased rate for the first ten to fifteen days and thereafter at a normal rate. The transfused sensitized cells remained sensitized during their life in the normal circulation.

3. The recipient's own cells became sensitized as a result of the transfusion of sensitized cells. Possible explanations are considered.

4. In acquired haemolytic anaemia the erythrocytes are sensitized, probably by a non-specific auto-antibody. The possible processes that may play a part in the rapid elimination of these sensitized cells are discussed.

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THE MICROBIOLOGICAL ASSAY OF RIBOFLAVIN AND NICOTINIC ACID IN URINE

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Riboflavin and nicotinic acid may be determined by (1) biological assay, using animals, (2) chemical methods, colorimetric or fluorometric, (3) microbiological assay, using bacteria of known nutritional requirements.

The biological properties of these two vitamins were established in animal experiments, and for a time similar assay methods were used to assess their concentrations in various types of materials. Such methods are time-consuming, require specialized experience, and are not particularly sensitive or accurate. Chemical methods which were developed later still lack sensitivity and are subject to inaccuracies owing to the necessity of a number of manipulations to remove interfering substances. Within recent years much information has been obtained with regard to the nutritional requirements of many species of bacteria. It has been shown that many require certain members of the vitamin B complex for optimum growth. When other nutritional requirements are present in excess, growth of a particular organism is proportional, within certain limits, to the concentration of an essential nutrient—e.g., a vitamin. This has resulted in the development of a wide variety of microbiological methods for the determination of vitamins, amino-acids, etc. Growth may be assessed either nephelometrically or, if the organism is an active acid-producer and the medium is highly buffered, by the amount of titratable acid produced. Such methods are extremely sensitive and little preliminary manipulative work is usually required.

The usual chemical methods for the determination of members of the vitamin B complex are, owing to their complexity, generally outside the scope of the hospital biochemical laboratory. Microbiological methods, on the other hand, owing to their greater simplicity, are more adaptable to hospital needs. Also multiple determinations with comparatively little extra labour may be carried out.

The determination of nicotinic acid and riboflavin in urine by microbiological methods has been examined. There have been many published methods, but those of Barton-Wright and Booth (1943) for riboflavin and Barton-Wright (1944, 1945) for nicotinic acid have with certain modifications proved to be satisfactory.

Procedure

Stock cultures are carried as stabs on the following medium:

Yeast extract	...	...	1.0 per cent
Glucose ...	...	...	0.5 per cent
Sodium acetate	...	...	0.6 per cent
Agar ...	...	...	1.5 per cent
pH ...	...	...	6.8

After inoculation, cultures are incubated at 37° C. for 48 hours and then preserved in a refrigerator. Stock cultures are renewed at fortnightly intervals.

TABLE I

Organism used	Vitamin
Lactobacillus helveticus (casei) N.C.T.C. No. 6375	Riboflavin
Lactobacillus arabinosus 17/5 N.C.T.C. No. 6376	Nicotinic acid

Normal urine contains a number of pyridine compounds closely related to nicotinic acid—namely, nicotinic acid, nicotinamide, nicotinuric acid, N¹ methylnicotinamide, and trigonelline. The response of *L. arabinosus* 17/5 to nicotinic acid, nicotinamide, and nicotinuric acid is proportional to their nicotinic acid content (Snell and Wright, 1941). The other two pyridine derivatives show no activity towards this organism.

The present writers have found that the dependence of their own cultures on these organisms for these vitamins has remained unchanged over a period of twelve months.

PREPARATION OF MEDIA

Stock Solutions.—The following stock solutions were prepared:

Photolysed peptone.—10 g. of peptone and 5 g. of sodium hydroxide are dissolved in 125 ml. water and placed in a narrow cylinder about 18 in. from a

100-watt electric lamp for two to three days. This procedure ensures the complete destruction of all riboflavin in the peptone. Then 7 ml. of glacial acetic acid and 3 g. of crystalline sodium acetate are added, the volume made up to 200 ml. with water and filtered.

Yeast supplement (riboflavin-free).—40 g. of basic lead acetate dissolved in 125 ml. of water are added to 25 g. of yeast extract in 125 ml. of water. The *p*H is adjusted to 10 with ammonia, and the precipitate filtered off. The filtrate after acidification with glacial acetic acid is treated with hydrogen sulphide gas until all the lead is precipitated. The lead sulphide is filtered off, excess hydrogen sulphide is removed by boiling, and the volume made up to 250 ml. by the addition of water.

Casein hydrolysate.—A spray-dried product has been used.

Cystine solution.—1 g. of cystine is added to 250 ml. water containing 1 ml. of concentrated hydrochloric acid and the mixture heated until all the cystine has passed into solution.

DL-*Tryptophan solution.*—1 g. of tryptophan is boiled with 250 ml. of water and concentrated hydrochloric acid added until solution is effected. (The synthetic DL-product should be used in preference to the natural L-tryptophan since the latter is often contaminated with vitamins.)

Adenine, guanine, and uracil solution.—0.1 g. of each are added to 100 ml. water containing a few drops of concentrated hydrochloric acid. Solution is effected by heat. The final volume should be made up to 100 ml. by the addition of water.

Xanthine solution.—0.1 g. is added to 100 ml. water containing a few drops of strong ammonia.

Calcium d-pantothenate solution.—0.1 g. is dissolved in 100 ml. water.

Nicotinic acid solution.—0.1 g. is dissolved in 100 ml. water. (1 ml. = 1,000 μg. nicotinic acid.)

Pyridoxine solution.—0.244 g. pyridoxine hydrochloride is dissolved in 100 ml. water.

p-*Aminobenzoic acid solution.*—0.1 g. is dissolved in 100 ml. water containing 1 ml. of glacial acetic acid.

Aneurin solution.—0.1 g. is dissolved in 100 ml. of 2 per cent hydrochloric acid.

Riboflavin solution.—25 mg. of riboflavin is added to a little water and 1 ml. of glacial acetic acid and diluted to 1,000 ml. with distilled water. This solution, owing to the sensitivity of riboflavin to light, should be preserved in a dark brown bottle. (1 ml. = 25 μg. riboflavin.)

Biotin solution.—An ampoule of 25 μg. biotin + 1 ml. of inorganic solution A is diluted to 250 ml. with water.

Inorganic solution A.—25 g. K_2HPO_4 and 25 g. KH_2PO_4 are dissolved in water and the volume made up to 250 ml.

Inorganic solution B.—10 g. $MgSO_4,7H_2O$, 0.5 g. $MnSO_4,4H_2O$, and 0.1 g. anhydrous $FeCl_3$ are dissolved in water containing a few drops of concentrated hydrochloric acid.

The solutions should be prepared with glass-distilled water and preserved in glass-stoppered bottles in a refrigerator. If a few drops of chloroform are added to each and assays are being carried out frequently, these solutions will keep well.

Media.—Sufficient for 100 tubes is prepared.

TABLE II

MEDIA PREPARED FROM STOCK SOLUTION AND OTHER SUBSTANCES

	Riboflavin assay	Nicotinic acid assay
Photolysed peptone solution ..	100 ml.	—
Spray-dried casein hydrolysate	—	6 g.
L-Cystine solution	25 ml.	50 ml.
DL-Tryptophan solution ..	25 ml.	50 ml.
Glucose	20 g.	20 g.
Xylose	1.0 g.	1 g.
Sodium acetate (hydrated) ..	—	33.0 g.
Riboflavin-free yeast supplement	20 ml.	—
Adenine, guanine, and uracil solution	10 ml.	10 ml.
Xanthine solution	10 ml.	10 ml.
Calcium *d*-pantothenate solution	1 ml.	1 ml.
Nicotinic acid solution ..	1 ml.	—
Pyridoxine solution	1 ml.	1 ml.
p-Aminobenzoic acid solution	4 ml.	1 ml.
Aneurin solution	—	1 ml.
Riboflavin solution	—	8 ml.
Biotin	—	4 ml.
Sodium chloride	5.0 g.	5.0 g.
Ammonium sulphate	3.0 g.	3.0 g.
Inorganic salt solution A ..	5.0 ml.	5.0 ml.
Inorganic salt solution B ..	5.0 ml.	5.0 ml.
Glass-distilled water to ..	500 ml.	500 ml.

The *p*H of the media is adjusted to 6.8 by the addition of sodium hydroxide solution using bromothymol blue as external indicator, or indicator test papers. After filtration through a No. 1 Whatman filter paper, a few drops of chloroform are added and the media preserved in a refrigerator. Under such conditions, especially if assays are carried out at frequent intervals, the prepared media will keep well.

Before use a portion of the medium should be heated to boiling point to remove chloroform.

Preparation of Assay Tubes.—Universal containers with metal caps and rubber seals were used in place of test tubes with cotton-wool plugs. After use tubes should be cleaned out with tap water and rinsed with glass-distilled water. It was found convenient to keep a set of tubes for one particular assay and to number the containers with a glass diamond.

In each series of assays blanks and a series of standards were prepared in duplicate. Although little

variation in the standard curves has been encountered over a period of twelve months, such controls are essential as a check on the non-varying dependence of the organism for the particular vitamin under investigation and the constancy of the medium.

Riboflavin.—Assay tubes-were prepared as follows :

Blanks.—5 ml. of water and 5 ml. of medium were measured into each tube.

Standards.—Standards containing 0.05, 0.10, 0.15, and 0.20 µg. of riboflavin were prepared.

The standard solution, 25 µg./ml., is diluted so that 1 ml. contains 0.05 µg. This solution should be prepared fresh before use.

TABLE III
QUANTITIES

Standard containing riboflavin (µg.)	Medium (ml.)	Dilute riboflavin standard solution (ml.)	Water
0.05	5	1	4
0.10	5	2	3
0.15	5	3	2
0.20	5	4	1

Urine.—1, 0.5, 0.25, and 0.1 ml. of urine diluted to 5 ml. were added to 5 ml. of medium. These quantities will cover all concentrations of riboflavin likely to be encountered in urine except when large quantities of riboflavin—for example, 10 mg.—have been administered. After oral administration of 10 mg. riboflavin, an additional 0.05, 0.025, and 0.01 ml. of urine diluted to 5 ml. should also be included.

Nicotinic Acid.—Assay tubes were prepared as follows :

Blanks.—5 ml. of water and 5 ml. of medium were measured into each tube.

Standards.—Standards containing 0.05, 0.10, 0.15, 0.20, 0.25. and 0.30 µg. of nicotinic acid were prepared. The standard solution, 1,000 µg./ml., is diluted so that 1 ml. contains 0.01 µg. nicotinic acid. This solution should be prepared fresh before use.

TABLE IV
QUANTITIES

Standard containing nicotinic acid (µg.)	Medium (ml.)	Dilute nicotinic acid standard solution (ml.)	Water
0.05	5	0.5	4.5
0.10	5	1.0	4.0
0.15	5	1.5	3.5
0.20	5	2.0	3.0
0.25	5	2.5	2.5
0.30	5	3.0	2.0

Urine.—0.5, 0.25, 0.1, and 0.05 ml. of urine diluted to 5 ml. were added to 5 ml. of medium. These quantities will cover all concentrations of nicotinic acid likely to be encountered in urine.

Filled tubes were sterilized by placing in a steamer or a hot-air oven at 100° C. for one hour. Autoclaving was found to produce intense brown colorations which rendered subsequent titration with alkali very difficult to assess.

INOCULATION

Riboflavin.—5 ml. of riboflavin medium and 5 ml. of distilled water containing 0.25 µg. of riboflavin were introduced into a universal container and sterilized as described above. The medium was inoculated with a portion of the stock agar stab culture by means of a sterile needle and incubated for 18 to 20 hours at 37° C. There should be good visible growth at the end of this period. The contents of the container were centrifuged, the supernatant fluid poured off, and 10 ml. of 0.85 per cent sterile saline solution added ; 20 drops of this suspension were mixed with 10 ml. of 0.85 per cent sterile saline solution, and 1 drop of this latter suspension added to each of the assay tubes.

Nicotinic Acid.—5 ml. of nicotinic acid medium and 5 ml. of distilled water containing 0.5 µg. of nicotinic acid were introduced into a universal container and sterilized as described above. It was inoculated with a portion of the stock agar stab culture by means of a sterile needle and incubated at 37° C. until good visible growth was observed. Usually this is attained after 18 hours, but on some occasions 36 hours' incubation is necessary. The contents of the container were centrifuged, the supernatant fluid poured off, and 10 ml. of 0.85 per cent sterile saline solution added ; 20 drops of this suspension were mixed with 100 ml. of 0.85 per cent sterile saline solution, and 1 drop of this latter suspension added to each of the assay tubes.

INCUBATION

Assay tubes were incubated at 37° C. for three days. Exact times are unnecessary since both unknowns and standards are subjected to the same treatment. At the end of the incubation period the lactic acid in the containers was titrated with 0.1 N-sodium hydroxide using bromothymol blue as indicator.

CALCULATION

A graph was constructed in which content of riboflavin (or nicotinic acid) in µg. was plotted against ml. 0.1 N-sodium hydroxide used in the respective titrations (see Figs. 1 and 2). Blanks should require less than 1 ml. of 0.1 N-sodium hydroxide to effect neutralization. The riboflavin (or nicotinic acid) content of the unknowns was then read off the graph using the titrimetric values. It has been found possible to obtain readings from at least two urinary levels,

differences never exceeding 15 per cent—i.e., between the limits of -

0.05–0.20 μg. riboflavin
and
0.05–0.30 μg. nicotinic acid.
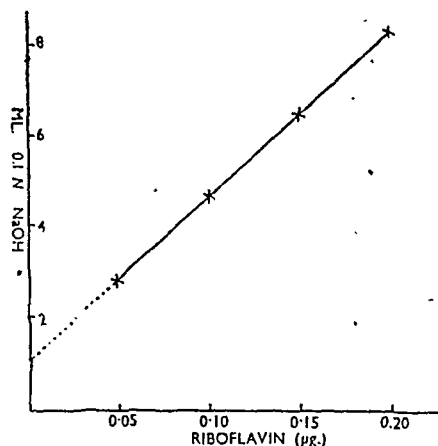
FIG. 1.—Assay of riboflavin.
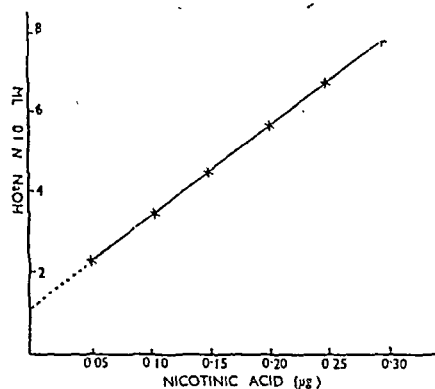
FIG. 2.—Assay of nicotinic acid.

More precise but more laborious methods of calculation have been suggested by Wood (1945, 1946). The normal excretion of these vitamins in urine is so wide and small differences at present of so little significance that in routine investigations there is little to be gained by such methods.

Experimental

Owing to the lability of riboflavin to light and alkali, urine passed over a period of 24 hours was collected in a dark brown bottle containing 5 ml. of glacial acetic acid. When it was necessary to keep the specimen for a longer period it was kept in a refrigerator. The stability of riboflavin and nicotinic acid in urine under such conditions was examined. Urines were assayed initially and 28 days later. From the results shown in Table V it will be seen that there was little change in the concentrations.

The effect of mild acid hydrolysis had little effect on the results (Table VI). (To obtain this hydrolysis 5 ml. + 0.5 ml. concentrated hydrochloric acid were placed in a boiling-water bath

TABLE V

STABILITY OF RIBOFLAVIN AND NICOTINIC ACID IN URINE

Riboflavin (Test No.)	Initially (μg. litre)	After 1 month's preservation (μg./litre)
1	825	800
2	825	850
3	400	420
4	600	650

Nicotinic acid (Test No.)	Initially (μg. litre)	After 1 month's preservation (μg./litre)
1	430	370
2	1,650	1,800
3	250	220
4	570	600
5	850	875
6	350	380

for one hour, cooled, and neutralized by the addition of sodium hydroxide.) This is in accordance with the fact that riboflavin is excreted in the free state and that nicotinic acid, nicotinamide, and

TABLE VI

EFFECT OF MILD ACID HYDROLYSIS ON THE MICROBIOLOGICAL ASSAY OF RIBOFLAVIN AND NICOTINIC ACID IN HUMAN URINE

	Before hydrolysis (μg./litre)	After acid hydrolysis (μg./litre)
Riboflavin	805	800
	170	137
	105	105
	70	60
	410	380
Nicotinic acid	1,100	950
	430	380
	430	480
	1,650	1,650
	570	540

nicotinuric acid exert equivalent responses towards *L. arabinosus* 17/5.

Recoveries of added riboflavin and nicotinic acid were found to be quantitative (Table VII). The initial content of riboflavin was found to be effectively removed by the addition of alkali and exposure to the light of a 100-watt electric lamp for four days. It was neutralized by the addition of hydrochloric acid.

In many of the chemical methods employed for the estimation of riboflavin and nicotinic acid,

TABLE VII

RECOVERY OF RIBOFLAVIN AND NICOTINIC ACID ADDED TO URINE

Test No.	Riboflavin added (μg./litre)	Riboflavin recovered* (μg./litre)	Test No.	Riboflavin added (μg./litre)	Riboflavin recovered* (μg./litre)	Test No.	Initially (μg./litre)	Nicotinic acid added (μg./litre)	Final (μg./litre)	Nicotinic acid recovered (μg./litre)
1	50	55	8	150	140	1	320	200	550	230
2	100	115	9	50	55	2	320	400	740	420
3	50	40	10	150	165	3	2,100	500	2,700	600
4	100	115	11	100	120	4	2,100	1,000	8,100	1,000
5	50	55	12	200	200	5	1,800	500	1,700	400
6	100	120	13	50	50	6	1,300	1,000	2,350	1,050
7	50	50	14	100	95	7	220	200	460	240
						8	220	400	650	430

* 1 mL. of of photolysed urine used in each recovery.

absorption on varied types of material is used as a preliminary to the final fluorometric or colorimetric evaluation. The effect of urinary deposits was investigated. Assays for riboflavin and nicotinic acid were carried out on the urine which had been well shaken, and on the supernatant fluid after centrifugalization. The results shown in Table VIII indicate that appreciable amounts of riboflavin were absorbed on the urinary deposits. Urinary deposits are variable and it is quite possible that such do exist and can absorb appreciable amounts of nicotinic acid. These experi-

TABLE VIII

ABSORPTION OF RIBOFLAVIN AND NICOTINIC ACID ON URINARY DEPOSITS

	Whole urine (μg./litre)	Centrifuged urine (supernatant μg./litre)
Riboflavin		
1	95	50
2	65	40
3	100	70
Nicotinic acid		
1	3,000	2,600
2	750	700
3	1,060	1,160

ments indicate that in vitamin B assays urines containing deposits should be so treated that representative samples may be measured.

Riboflavin 10 mg. and nicotinamide 50 mg. were administered to patients who had no evidence of deficiency of these vitamins.* Excretion of these vitamins before and during the first 24 hours after administration was determined (Table IX). Increased excretion of riboflavin accounted for

* The test dose also included 5 mg. aneurin and 200 mg. ascorbic acid.

approximately 50 per cent of the administered vitamin. This is in accordance with the findings of Melnick *et al.* (1945), who used fluorometric methods of analysis. There was little change in the excretion of nicotinic acid. This is in accor-

TABLE IX

RIBOFLAVIN AND NICOTINIC ACID

Case No.		Riboflavin (μg./diem)	Nicotinic acid (μg./diem)
1	Before test dose	920	0.84
	After ,, ,,	6,030	0.34
2	Before test dose	570	0.76
	After ,, ,,	4,374	1.38
3	Before test dose	468	0.40
	After ,, ,,	5,890	0.53
4	Before test dose	450	1.17
	After ,, ,,	4,480	0.57
5	Before test dose	200	0.97
	After ,, ,,	4,500	1.12

dance with the findings of Melnick *et al.* (1940) and Huff and Perlzweig (1943) that, following ingestion of nicotinamide, N^1 methylnicotinamide is the main metabolite excreted in the urine.

Summary

Factors that influence the microbiological determination of riboflavin and nicotinic acid derivatives in urine have been investigated.

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L.

THE DETERMINATION OF THE TOTAL NEUTRAL 17-KETOSTEROIDS IN URINE

BY

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Callow and others (1938) appear to be the first to have produced a reasonably accurate method for the estimation of 17-ketosteroids by means of the Zimmermann reaction. The method is, however, somewhat laborious and time-consuming. This work was followed by many papers in which modifications in technique are described. Tompsett and Oastler (1946) have reviewed the literature and described a method which in comparison with many others is comparatively simple and is suitable for use in the busy hospital laboratory. In this method, the ketosteroids are liberated by acid hydrolysis and then extracted with ether, the final result being obtained colorimetrically with the Zimmermann reaction. This method gave essentially the same results as that of Callow and others. Tompsett and Oastler (1948) have examined the neutral steroid fraction of human urine and used acid hydrolysis coupled with simultaneous extraction with toluol to effect separation. It was found that this method gave essentially the same results for total 17-ketosteroids as the earlier method of separation. It would appear that the nature of the extractant and even possibly the exact time of hydrolysis affects the true value of the total 17-ketosteroids but little.

When applied to urinary extracts, the Zimmermann reaction produces a purple colour with 20-ketosteroids—for example pregnanolones—as well as with 17-ketosteroids. Venning (1946) has stated that pregnanolones produce only one-eighth of the colour produced by a corresponding amount of 17-ketosteroid. 20-Ketosteroids are only found in appreciable quantities in pregnancy and possibly in adrenal cortical tumour and hyperplasia (Tompsett and Oastler, 1948 ; Tompsett, 1949). Tompsett (1949) has described a technique by which the presence of 20-ketosteroids in appreciable quantities may be detected.

The greatest disadvantage of the Zimmermann reaction is that in some cases an unspecific brown colour is encountered. Many attempts have been made to eliminate this or correct for it. The use of corrections which involve readings from two colour filters has been suggested. Elimination of the brown interfering pigment may be achieved by the use of Girard's reagent T, which forms water-soluble derivatives with the ketone fraction of the neutral steroids. By suspension in water and extraction with ether, non-ketonic materials are removed. On treatment with hydrochloric acid, free ketosteroids are liberated and may be extracted with ether. It has been supposed that the materials responsible for the unspecific brown colours are removed in the first ether extracts—that is, in the non-ketonic fractions.

The writer has, however, noted that a brown-coloured insoluble material is located in varying amounts at the ether/aqueous interface, and he believes that this is mainly responsible for the unspecific brown colorations. Production of unspecific brown colorations in the Zimmermann reaction is much less frequent with ether than with toluol extraction. Such brown colorations are probably due to derivatives of insoluble soaps of fatty acids which are the main constituents of the brown scum that separates at the ether/aqueous interface during the course of the Girard T reagent technique. The final colorimetric evaluation of colour is most suitably carried out in the Spekker absorptiometer. Visual inspection should always be carried out. (The human eye is still one of the best optical discriminating instruments, a fact that is often forgotten in this mechanical age.) The writer prefers to use Spekker green filter 5 to Ilford Spectrum filter No. 604. The former permits of more rapid readings and possesses similar sensitivity.

Alcoholic solutions of the urinary steroids are invariably coloured purple or yellow or intermediate shades. The purple colour of the original extract disappears during the production of the

purple of the Zimmermann reaction, but the yellow colours are unaffected. It is necessary to carry out blanks.

The following is a detailed description of the procedure now recommended for use in hospital biochemical laboratories.

Procedure

REAGENTS

1. *Concentrated hydrochloric acid* (analar reagent).

2. *Peroxide-free ether.*

3. *Ten per cent aqueous sodium hydroxide.*

4. *Aldehyde-free absolute alcohol.*—This is prepared in the following way: 4 g. of *m*-phenylenediamine hydrochloride are added to 1 litre of commercial absolute alcohol and the mixture is allowed to stand in the dark for one week, with occasional shaking. The alcohol is distilled in an all-glass apparatus. The distilled alcohol should be preserved in a dark bottle in a dark cupboard.

5. *Two per cent* m-*dinitrobenzene in aldehyde-free alcohol.*—Even the best commercial specimens of *m*-dinitrobenzene contain impurities which interfere seriously with the final colour reaction. The best commercial specimen should be purified in the following way: 20 g. are dissolved in 750 ml. of 95 per cent ethyl alcohol in an 8-litre flask and warmed to 40° C. Aqueous 2N-sodium hydroxide solution is then added until there is no further increase in the pink colour. After 5 minutes the solution is cooled and about 2.5 litres of water are added rapidly and the mixture stirred well. The precipitated *m*-dinitrobenzene is collected on a Buchner funnel, washed thoroughly with large quantities of water, and recrystallized twice in succession from about 120 ml. and 80 ml. respectively of absolute alcohol. A mixture of equal volumes of a 1 per cent alcoholic solution of *m*-dinitrobenzene with aqueous 5N-potassium hydroxide solution should develop no colour after standing for 1 hour. For use a 2 per cent solution in *aldehyde-free* absolute alcohol is prepared. The solution should be preserved in the dark. (The purification procedures described for absolute alcohol and *m*-dinitrobenzene are those recommended by Callow and others, 1938, 1939).

6. *5N-potassium hydroxide* (analar) in water.

7. *Commercial absolute alcohol.*

8. *Glacial acetic acid.*

9. *Thirty per cent sodium hydroxide.*

GLASSWARE

All glassware should be scrupulously clean. Apparatus consists of: (1) 500-ml. glass-stoppered separating funnels, (2) 250-ml. and 1-litre conical flasks (Pyrex), (3) 500-ml. beakers, (4) 50-ml. glass-stoppered cylinder, and (5) Pyrex test tubes (130 × 15 mm.); before use, these are cleaned with nitric acid and chromic acid mixture, then washed with water followed by absolute alcohol, and finally dried in an oven.

TECHNIQUE

Preparation of Extract.—A twenty-four-hour specimen of urine is collected in a Winchester quart bottle containing 10 ml. concentrated hydrochloric acid. 250 ml. are boiled gently with 25 ml. concentrated hydrochloric acid in a litre conical flask over a bunsen burner for 10 minutes. The flask and its contents are then immediately cooled in a dish of running water, and the hydrolysed urine is transferred to a 500-ml. separating funnel where it is extracted three times with 80-ml. portions of peroxide-free ether.

The pooled ether extracts are washed twice with 80-ml. portions of 10 per cent sodium hydroxide and twice with 80-ml. portions of water. The ether extract is then evaporated to dryness in a 250-ml. conical flask and heated in a hot-air oven at 100° C. for a few minutes.

The dry steroid residue is dissolved in 0.5 ml. glacial acetic acid, and 30 ml. of water added; 1 ml. of 30 per cent sodium hydroxide is added and the mixture transferred to a 50-ml. glass-stoppered cylinder. The aqueous solution is extracted three times with 20-ml. portions of peroxide-free ether. The ether layer which is removed with a teat-pipette is transferred to a 250-ml. conical flask. Care should be taken that the brown amorphous material which settles at the aqueous-ether interface is not disturbed in this process. The ether extract is evaporated to dryness and then heated in a hot-air oven at 100° C. for a few minutes. Dried steroidal extracts appear to keep indefinitely.

Colorimetric Estimation.—The colour is developed in Pyrex test tubes (130 × 15 mm.) which must be scrupulously clean. The dry steroidal extract is dissolved in 5 ml. aldehyde-free absolute alcohol. Three tubes are required for an estimation:

1. *Unknown.*

   0.2 ml. of alcohol solution of urinary steroids;
   0.2 ml. of 2 per cent alcoholic solution of *m*-dinitrobenzene;
   0.2 ml. of 5N-aqueous potassium hydroxide.

2. *Urine blank.*

   0.2 ml. alcohol solution of urinary steroids;
   0.2 ml. of aldehyde-free alcohol;
   0.2 ml. of 5N-aqueous potassium hydroxide.

3. *Reagent blank.*

   0.2 ml. aldehyde-free alcohol;
   0.2 ml. of 2 per cent alcoholic solution of *m*-dinitrobenzene;
   0.2 ml. of 5N-aqueous potassium hydroxide.

The tubes are placed in a water-bath at a temperature of 25° C. for a period of 45 minutes and shielded from direct light. After incubation 10 ml. of 67 per cent aqueous alcohol (67 ml. absolute alcohol to 100 ml. with distilled water) are added to each and their contents mixed. Absorption is measured in a Spekker absorptiometer with Spekker green filters 5 in posi-

tion. The small absorptions due to the urine and reagent blanks are deducted from the estimation (1) and the quantity of 17-ketosteroid present read off from a standard curve in terms of androsterone.

In the event of the urine containing more than 20 mg./litre, it will be necessary to dilute the alcoholic extract further—for example, to 10 or 20 ml.

Standard Curves.—A suitable standard curve may be prepared from *0.05, 0.10, 0.15,* and *0.20 mg.* androsterone in 0.2 ml. alcohol.

Dehydro*iso*androsterone, when available, gives an almost identical curve.

Oestrone, a phenolic 17-ketosteroid which is removed from urinary extracts by the alkali treatment, gives a similar curve. The colour with oestrone is approximately 5 per cent greater than with a corresponding amount of androsterone.

Results

Recovery experiments are obviously of little value in an investigation of this type. Results obtained by the procedure described above have been compared with those obtained after complete Girard T reagent separation of the ketones from an extract obtained after acid hydrolysis coupled with simultaneous extraction with tuluol. As seen from the Table the figures are similar.

Summary

A method is described for the determination of the total neutral 17-ketosteroids in urine. It is suitable for the routine laboratory and includes a procedure for the elimination of undesirable brown colorations which sometimes interfere in the final colorimetric evaluation.

TABLE

COMPARISON OF 17-KETOSTEROID VALUES OBTAINED BY GIRARD T SEPARATION AND BY ETHER EXTRACTION

Urine sample	17-Ketosteroids (mg./day)	
	A	B
1	3.4	3.8
2	11.6	11.1
3	13.7	12.4
4	13.3	14.5
5	19.2	20.4
6	19.5	19.5
7	19.8	20.4
8	21.7	23.6
9	29.0	28.0
10	35.0	37.5

A = Acid hydrolysis with simultaneous extraction with toluol; ketones separated with Girard reagent T. B = Acid hydrolysis followed by ether extraction.

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THE VALUE OF DILUTED ANTIGEN IN THE WASSERMANN REACTION

BY

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This paper reports an attempt to assess the value of a modification of the Wassermann reaction wherein a suitable dilution of the normal antigen together with an increased amount of serum was used in an additional tube as suggested by Richardson (1940). By this method we hoped, first, to be able to differentiate specific reactions in sera giving doubtful results with the standard Wassermann technique from non-specific reactions, and, secondly, to detect weakly positive sera in cases of treated syphilis.

Sordelli (1931), by using two strengths of lipoidal emulsion as antigen in combination with two strengths of serum, showed that the specificity of the Wassermann reaction could be greatly increased. Richardson (1940) adapted the Wyler-Harrison technique accordingly and improved the specificity of the Wassermann reaction in doubtful cases and at the same time maintained the sensitivity as compared with the standard Wyler-Harrison technique.

Vaughan (1947) recently published results agreeing with those of Richardson and found that both the specificity and sensitivity were increased by the new method. Both Richardson and Vaughan compared the standard Wassermann reaction with the modified technique whereas we have used this method as a verification test for doubtful sera and as a more sensitive test for weakly positive sera.

The theoretical basis of the modified test as suggested by Richardson is as follows. There is abundant excess of specific hapten present in the lipoid extract; therefore with weakly reacting sera the excess of specific hapten in the usual antigen (1 : 15 dilution of the lipoid extract) might produce zonal inhibition which would be overcome by using a diluted antigen (1 : 90 to 1 : 150 dilution of the lipoid extract). Combining proportions of antigen and antibody would thereby be nearer the optimum. Further, at such high dilutions the strength of any contaminating non-specific hapten or haptens should be too low to fix a full dose of complement. So, by using the usual antigen in one tube and in another the antigen diluted 1 : 6 to 1 : 10, the specific and non-specific reactions should be more easily distinguished. A specific reaction which is partly suppressed by zone inhibition with the usual antigen should be enhanced with the diluted antigen where proportions are nearer the optimum, whereas a non-specific reaction which is partial with the usual antigen should be weakened with the diluted antigen, provided that any contaminating hapten is not present in sufficient strength to fix a full complement dose when the antigen is diluted.

Technique

The Wassermann Reaction.—The technique used for the routine test was that described by Panton and Marrack (1945). To this we added tube 4, which contained diluted antigen and increased serum. The full test is summarized in Table I.

TABLE I

SUMMARY OF WASSERMANN TEST

Tube	Serum (ml.)	Saline (ml.)	Complement (3 M.H.D. in 0.5 ml.)	Antigen (ml.)	Sensitized cells (ml.)
1	0.1	0.4	0.5	0.5	1.0
2	0.1	0.2	0.7	0.5	1.0
3 (Control)	0.2	0.8	0.5	0	1.0
4	0.2	0.3	0.5	0.5 (diluted)	1.0

Sera.—The sera were heated at 56° C. for half an hour just before the test.

Antigen.—The antigen was that described by Panton and Marrack (1945), but in tube 4 it was diluted 1 in 7 with normal saline. This was the dilution found by titration to be the most satisfactory for the batch of antigen used.

Control Tube.—When tube 4 was not in use, tube 3 contained 0.1 ml. of serum and 0.9 ml. of saline, but as 0.2 ml. of the serum were used in tube 4 the control tube had to contain a similar amount with 0.8 ml. of saline to cover any anticomplementary action in tube 4.

Sensitized Cells.—The sensitized cells were an equal mixture of 5 per cent sheep red blood cells and 0.5 per cent haemolytic serum (B.W.) in normal saline.

Incubation.—The tests were incubated at 37° C. for 45 minutes at the fixation stage, and after the addition of the cells for a further 20 minutes at 37° C.

The Kahn Reaction.—All sera were tested at the same time by the standard Kahn reaction.

Reading of the Tests.—The Kahn tests were read as positive, negative, or doubtful. The Wassermann reactions were read similarly; that is, in the absence of complete fixation or complete haemolysis in all test tubes the result was doubtful. After the tests were read dilutions from the control tubes which · represented 100 per cent haemolysis were made with water in four similar tubes to give standards of 20, 40, 60, and 80 per cent haemolysis (that is, 80, 60, 40, and 20 per cent of fixation). All tubes not showing complete haemolysis were centrifuged and the supernatant was compared with the five standard tubes. The degree of haemolysis in tubes 1, 2, and 4 of all tests was thereby estimated to the nearest 10 per cent and recorded.

Selection and Classification of Cases

During the investigation approximately 9,000 routine standard Wassermann and Kahn reactions were carried out. The sera came from venereal disease and antenatal clinics and from general cases. During the latter part of the investigation we ceased to do the Wassermann and standard Kahn reactions on the antenatal cases as a routine, but instead used a presumptive Kahn reaction as a screening test. Sera with doubtful or positive Kahn reactions were also received from other laboratories in the region which did not undertake Wassermann reactions.

From these routine cases we selected for the modified test: (1) those sera showing incomplete fixation with 3 M.H.D. of complement; (2) those sera showing a discrepancy between the routine Wassermann and Kahn reaction; (3) sera from cases of treated syphilis which previously had not been consistently negative with the modified test; (4) sera from cases giving positive routine Wasser-

mann or Kahn reactions with no clinical evidence of syphilis; and (5) sera from antenatal clinics giving a positive or doubtful presumptive Kahn reaction.

These cases were classified as follows: group I, untreated syphilis; group II, treated syphilis; group III, cases suspected of syphilis from the history or clinical signs; and group IV, cases without history or signs of syphilis.

The total number investigated was one thousand.

If no tube 4 had been used our Wassermann reaction results would have been as shown in Table II.

TABLE II

RESULTS OF THE WASSERMANN REACTION BY THE STANDARD TECHNIQUE

Group	No. of sera	Numbers			Percentages		
		Posi-tive	Doubt-ful	Nega-tive	Posi-tive	Doubt-ful	Nega-tive
I	68	44	13	11	65	19	16
II	531	95	137	299	18	26	56
III	140	20	23	97	14	17	69
IV	261	3	19	239	1	7	92

It was expected that positive results would occur with decreasing frequency in our clinical groups I to IV, and, as Table II shows, our belief was amply confirmed. When the negative results by the standard technique—i.e., showing complete haemolysis in tubes 1 and 2—were considered in the light of haemolysis or fixation in tube 4 it was found that some were confirmed by complete haemolysis in tube 4 and others were made doubtful by the presence of complete or partial fixation in that tube (see Table III).

TABLE III

NEGATIVE RESULTS (100 PER CENT HAEMOLYSIS IN TUBES 1 AND 2).

Group	Total tests	Confirmed by tube 4	Made doubtful by tube 4	Percentage made doubtful
I	11	5	6	54.5
II	299	216	83	27.8
III	97	88	9	9.3
IV	239	237	2	0.8

This shows that of 646 sera which would have been negative with the standard Wassermann reaction technique, 100 became doubtful by the

modified technique. As 89 of these are known syphilitic cases and nine probably or possibly syphilitic, there is an obvious increase in sensitivity.

Turning now to the doubtful results by the standard technique and assessing them with the results in tube 4, they are more clearly seen if those showing fixation in tube 4 equal to or greater than in tube 1 are grouped together, and those with fixation in tube 4 less than in tube 1 kept separate.

TABLE IV

DOUBTFUL RESULTS (PARTIAL FIXATION IN TUBES 1 AND 2)

Group	No. of sera	Fixation		Percentages	
		$4 \geqslant 1$	$4 < 1$	$4 \geqslant 1$	$4 < 1$
I	13	13	0	100	0
II	137	111	26	81	19
III	23	14	9	61	39
IV	19	3	16	16	84

It is clear that fixation is less in tube 4 than in tube 1, with increasing frequency from group 1 to group IV, and suggests that this fact is helpful in differentiating between those irregular results due to syphilis from those which are not, thereby increasing the specificity of the test. In only five cases was the standard Wassermann positive (that is, full fixation in tubes 1 and 2) converted to doubtful by the presence of tube 4 (partial fixation in that tube). In all, the fixation in this tube was high—namely, 80 per cent, 80 per cent, 70 per cent, 60 per cent, 60 per cent. The significance of these results is discussed later. It is evident that the addition of tube 4, and the use of this reading in accordance with theoretical expectation, improves the specificity and sensitivity of the Wassermann reaction.

In order to combine the haemolysis readings so as best to separate probably positive from probably negative results, it is necessary to examine the figures without preconceptions. The statistical methods used are described more fully elsewhere (Jennison, Penfold, and Roberts, 1948); in the present paper we are presenting briefly the results obtained. The material comprises the readings in the three tubes for the 297 cases in which haemolysis is incomplete in at least one tube. The problem is to find that method of using the readings which maximizes the variation between the four clinical groups, and at the same time minimizing the variation within the groups; in other words, what is required is to combine the percentage haemolysis readings so as to make the four clinical groups as unlike as possible. The measure of the efficiency attained is the ratio of the variation between groups to the variation within groups; this is called the variance ratio.

It was found that the percentage haemolysis figures in tubes 1 and 2 were, when taken alone, of no value for classifying doubtful sera, for the variation within groups actually exceeds the variation between groups. (This is an interesting confirmation of the sound but not always accepted practice that the standard Wassermann reaction should not be read otherwise than as positive, doubtful, and negative.) The readings in tube 4, however, gave a very different result. The variance ratio is no less than 12.10, indicating a very useful amount of discrimination. This is illustrated graphically in Fig. 1, which shows that the
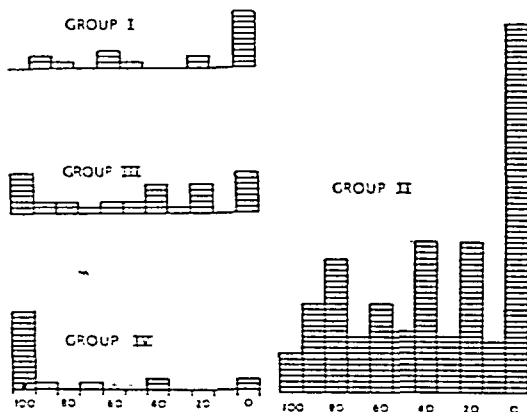
FIG. 1

less haemolysis there is in tube 4 the more likely the result is to be truly positive (using, as always, the criterion that the four clinical groups represent decreasing proportions of true sero-positive syphilis). Actually the reading in tube 4 alone differentiates the groups somewhat better than does the difference between tubes 1 and 4, the method suggested by theory. This latter method gives a variance ratio of 10.67. The discrimination attained is illustrated by Fig. 2.

The next task was to combine the readings in all the tubes in such a way as to maximize the difference between groups. The function which does so is the following:

$105 - (\%$ haemolysis in tube 1$) - \frac{1}{2}(\%$ haemolysis in tube 2$) - 1\frac{1}{2}(\%$ haemolysis in tube 4$)$

FIG. 2

as " probably positive " and a negative answer as " probably negative " as many group II as possible are made probably positive without making more than five of group IV probably positive. The discrimination attained is illustrated in Fig. 3.

The result is a remarkable one. Although percentage haemolysis in tubes 1 and 2 is useless by itself for further discrimination of doubtful sera, yet in combination these readings are capable of improving considerably the result obtained from tube 4 alone. Furthermore the function confirms theoretical expectation, for its leading feature is the difference between tube 1 and tube 4. Its greater efficiency compared with the simple difference method is due to assigning greater weight to tube 4, to taking into account the magnitude of the difference, and also to making some lesser use of tube 2. Actually, the increased efficiency attained by using the discriminant function is not fully shown by the comparison of the variance ratios because of the limited range of values when readings are made in steps of 10 per cent.

It will be noted that as in the difference method (tube 1—tube 4) a previously negative result made doubtful by tube 4 becomes probably positive, but unlike the difference method, the function makes the five previously full positives probably positive. In all the fixation in tube 4 was high (80 per cent, 80 per cent, 70 per cent, 60 per cent, 60 per cent) ; it would have to be 20 per cent or less to make such a result " probably negative." As three of these cases belonged to group II and two to group III this would appear to be an additional advantage of the function.

A comparison of the difference method and the discriminant function method is shown in Table VI.

The variance ratio is now 16.77—that is, the variation between the four clinical groups is 16.77 times the variation within the groups.

TABLE V
COMPARISON OF EFFICIENCY OF DIFFERENT PROCEDURES

	Variance ratio	Relative efficiency
Discriminant function	16.77	100
Reading in tube 4 alone	12.10	72
Difference tube 4–tube 1	10.67	64
Tubes 1 and 2 alone ..	<1	—

TABLE VI
COMPARISON OF THE DISCRIMINANT FUNCTION AND SIMPLE DIFFERENCES WITH 297 DOUBTFUL SERA

Group	Probably positive		Probably negative	
	Function	Difference	Function	Difference
I	19	19	0	0
II	212	194	11	29
III	27	23	7	11
IV	5	5	16	16

The figures under " Difference " are obtained by adding 4>1 and 4=1 for the " probably positive " and 4<1 for the " probably negative."

The actual magnitude of the figure given by the function with individual sera should not be inter-

The rounding off of the coefficients of the function to 1, $\frac{1}{2}$, and $1\frac{1}{4}$ entails only a trifling loss of information and has the great advantage of giving a formula that can be worked out in a moment. The arbitrary constant, 105, has been chosen so that if a positive answer (including zero) is regarded

FIG. 3
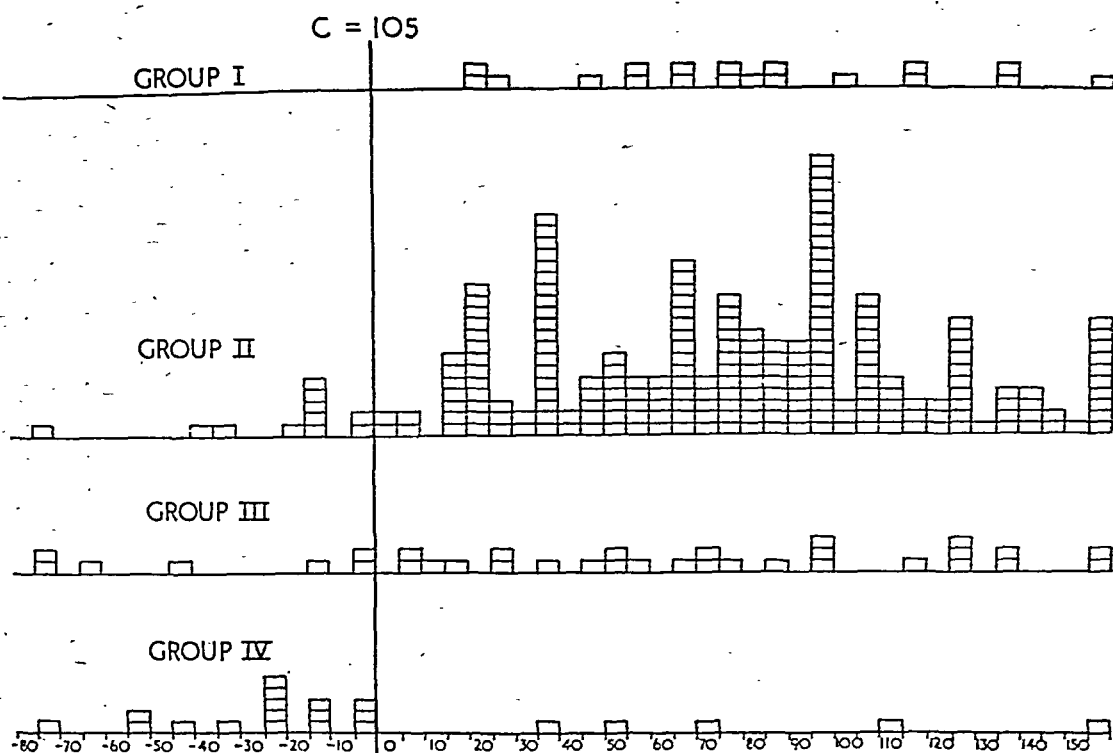
preted too closely ; that is to say, while it may be true in a rough general way that the larger the figure the "more positive" the result and vice versa, there will be many anomalies. This is due to the very non-normal distribution of the haemolysis figures. In spite of this a straightforward linear function of the variables is obviously useful, and a more complex function might well prove no more satisfactory, and would be both difficult and tedious to apply in practice. It is clear that common sense must be used with the results obtained, caution observed in arguing from actual figures, and results near the borderline read with some degree of reserve.

No attempt to assess the value and position of the standard Kahn reaction was undertaken. This has already been done many times. However, in terms of simple product-moment correlation coefficients, using three categories, positive, doubtful, and negative, the correlation of the Kahn test with the various tubes was as follows:

Tube 1	...	...	...	0.6289
Tube 2	...	...	...	0.6023
Tube 4	...	...	...	0.6766

The closer correspondence of tube 4 with the Kahn is not very marked, but the difference is significant.

Summary

One thousand Wassermann and Kahn tests were performed on sera expected to give difficult results. The sera were separated into four groups according to the clinical diagnosis. The standard Kahn test was used, but the Wassermann was modified by the addition of an extra tube (tube 4) in which was increased serum and decreased antigen. The addition of this tube to the test increased its specificity and sensitivity. A discriminant function was worked out and used with the results to aid their interpretation. Its value has been shown and it is simple to use. The Kahn test correlated most closely with the additional tube.

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COLIFORM INFECTION OF THE URINARY TRACT

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This paper concerns the investigation of the types of coliform organisms encountered in the urines of one hundred consecutive cases of pyuria admitted to Townleys Hospital, Bolton, during 1948. Recently Warner (1948), discussing urinary infection in paraplegic patients, has drawn attention to the frequency with which *Bact. aerogenes* was found. This organism was insensitive both to sulphanilamide and to penicillin, and Warner considered that these drugs may be of only limited value in urinary infection. It was thought that it would be interesting to discover if a similar high incidence of *Bact. aerogenes* would be found in other types of urinary infection and to consider all the types of coliform organisms found from the point of view of chemotherapy.

Methods

Wet and stained films of centrifuged deposits from catheter specimens of the urines were examined and the urines cultured on MacConkey's medium and blood agar. Any coliform bacilli isolated were identified by biochemical tests, and their sensitivity to sulphanilamide, penicillin, and streptomycin was determined. The fermentation reactions using glucose, mannitol, lactose, sucrose, and salicin were employed. Other tests used were: the formation of indole, gas production at 44° C., utilization of citrate, Vosges Proskauer test (Barritt's modification), and the methyl red reaction. The coliform bacilli were identified by the criteria described in Topley and Wilson's *Principles of Bacteriology and Immunity* (third edition).

Of the eighteen strains of *Bact. aerogenes* tested, six produced gas at 44° C. after 48 hours' incubation though not after 24 hours'. They were, however, classified as *Bact. aerogenes*. *P. vulgaris* was distinguished from *P. morgani* by the ability to swarm on blood agar. The paracolon bacilli included three anaerogenic strains, two of which were late lactose fermenters.

Sulphanilamide sensitivity tests were carried out by the method of Harper and Cawston (1945). Penicillin and streptomycin sensitivities were determined by inoculating one drop of an overnight broth culture of the organism under test into broth containing the drug in the appropriate concentration. These cultures were incubated overnight and examined for growth next morning.

Results

Table I shows the incidence of the varieties of coliform and other organisms associated with various clinical conditions. The cases were divided into two groups: group 1, in which there was no gross lesion of the urinary tract; and group 2, where infection was secondary to a surgical or medical condition affecting the tract.

It was found that in group 1 *Bact. coli* was the predominant organism (49 strains compared with 12 of other organisms) whereas in group 2 *Bact. coli* was relatively uncommon (7 strains compared with 56 of other organisms). Its place was taken in roughly equal proportions by *P. vulgaris*, *P. morgani*, and *Bact. aerogenes*. In group 1 pure cultures were frequent and Gram-positive cocci infrequent; in group 2 the reverse was found.

Table II shows the sensitivities to sulphanilamide, penicillin, and streptomycin. In planning these tests regard was paid to the concentrations that could be obtained in the urine. The Medical Research Council pamphlet suggests that concentration of sulphanilamide of 100 mg./100 ml. of urine can readily be obtained. Peeney (1946) found that concentrations of up to 200 units per ml. of penicillin could be maintained in the urine, while Petroff and Lucas (1947) obtained average concentration of 70 to 100 µg. of streptomycin per ml. Table II shows that bacteriostatic concentration to *Bact. coli* can be obtained in the urine both by sulphanilamide and by penicillin, to *P. vulgaris* by penicillin, and to a few strains (4 out of 19) by sulphanilamide. Bacteriostatic concentrations could not be obtained with either of these two drugs against *Bact. aerogenes* or *P. morgani*. All but one of the coliform strains tested were sensitive to streptomycin.

TABLE I

VARIETIES OF COLIFORM AND OTHER ORGANISMS ASSOCIATED WITH VARIOUS CLINICAL CONDITIONS

		No. of cases	Bact. coli	Bact. aerogenes	P. vulgaris	P. morgani	Atypical Bact. coli	Paracolon bacilli	Ps. pyocyanea	Bact. alkaligenes	Gram-positive cocci	Pure culture
Group 1: Cases without gross lesion of urinary tract	Acute pyelitis with fever and pain:											
	(a) pregnancy	16	16	—	—	—	—	—	—	—	—	16
	(b) non-pregnant	5	5	—	—	—	—	—	—	—	—	5
	Pyelitis of pregnancy without fever or pain	11	10	1	—	—	—	—	—	—	—	11
	Pyelitis in children	2	1	—	—	—	1	—	—	—	—	2
	Cystitis of pregnancy	7	2	—	1	—	2	3	—	—	2	4
	Cystitis of puerperium	8	8	—	—	—	—	—	—	—	4	4
	Cystitis	11	7	—	2	—	—	1	—	1	5	6
	Total, Group 1	60	49	1	3	—	3	4	—	1	11	48
Group 2: Cases with surgical or medical lesion affecting the urinary tract	Enlargement of prostate	29	4	14	14	13	—	2	3	—	23	5
	Paraplegia	7	3	2	2	1	—	—	—	—	5	1
	Papilloma of bladder	2	—	1	—	—	1	—	—	—	1	1
	Extravasation of urine	1	—	1	—	—	—	—	—	—	—	1
	Vesicovaginal fistula	1	—	—	1	—	1	—	—	—	1	—
	Total, Group 2	40	7	18	17	14	2	2	3	—	30	8

In group 2 there were two females and in group 1 three males: two young men with acute pyelitis and one child with pyelitis.

TABLE II

CONCENTRATION OF DRUG TO WHICH ORGANISMS ARE SENSITIVE

	Penicillin units per ml.				Sulphanilamide mg. per 100 ml.					Streptomycin µg. per ml.							
	No. of strains tested	Not sensitive >500	500 to 100	Less than 100	No. of strains tested	Not sensitive >200	200 to 100	100 to 20	Less than 20	No. of strains tested	1,000 to 500	100 to 50	50 to 25	25 to 10	10 to 5	5 to 2.5	Less than 2.5
Bact. coli	56	1	2	53	56	1	2	22	31	27	—	—	—	2	8	15	2
Bact. aerogenes	19	19	—	—	19	17	—	2	—	12	—	—	—	—	1	7	4
Proteus vulgaris	20	—	—	20	19	15	—	1	3	16	—	1	15	—	—	—	—
P. morgani	14	13	1	—	12	11	—	1	—	10	1	—	2	1	1	5	—
Atypical *Bact. coli*	5	1	2	2	5	—	—	4	1	4	—	—	—	—	—	4	—
Ps. pyocyanea	3	3	—	—	3	3	—	—	—	3	—	—	2	1	—	—	—
Paracolon bacilli	6	1	3	2	6	—	1	4	1	3	—	—	1	—	1	1	1
Bact. alkaligenes	1	—	—	1	1	—	—	—	1	1	—	—	—	—	—	—	1

A number of sensitive organisms were tested at concentrations of penicillin 50 units ml. Out of 31 strains of *Bact. coli* tested 23 strains showed a good growth, 6 strains showed some inhibition, and 2 strains were completely inhibited. Out of 14 strains of *P. vulgaris* tested, 4 gave a good growth in 50 units/ml. penicillin, 8 showed some inhibition, and 2 were completely inhibited.

Discussion

Reports vary of the incidence of the different types of coliform bacilli in urinary infections. Thus in 200 cultures from all clinical types of infection Hill *et al.* (1929) found 100 strains of *Bact. coli*, 79 strains of *Bact. aerogenes*, and 5 strains of the proteus group. In the urine of paraplegic patients Petroff and Lucas (1946) found 41% of the coliform organisms were *Bact. aerogenes* and 16% proteus, while Warner (1948), considering only those which fermented lactose, found 32 out of 50 coliform organisms studied were *Bact. aerogenes*. On the other hand, Cross (1937), in a series from which urinary infections secondary to prostatic enlargement were excluded, found 17 strains of *Bact. coli*, 2 of *Bact. aerogenes*, and 5 of other organisms. Marple (1941) in a series of thirteen cases of women with pyuria found *Bact. coli* strains only, and Dodds (1931), considering urinary infection in pregnancy, found 49 *Bact. coli* strains and only one *Bact. aerogenes* (*Bact. friedlander*).

The present findings suggest that the rate of urinary flow may be the factor which determines the incidence of *Bact. coli* in infected urines. In this series the group of cases of primary urinary infection consists largely of women and includes a high proportion of cases occurring in pregnancy, whereas the group of cases of infections secondary to obstruction includes a large proportion of cases with enlargement of the prostate. No doubt this distribution of cases is determined in part by the type of case admitted to the particular hospital, but it is thought that any representative series of cases of urinary infection would show that the primary cases were much commoner in women than in men, that in women they were often associated with pregnancy, and that the commonest cause of urinary obstruction was enlargement of the prostate (and for this reason the group where the infection was secondary to obstruction would include many cases of this condition). The series is considered, therefore, representative enough to draw general conclusions.

A possible explanation of the difference in distribution of *Bact. coli* infections between the two groups might be that in the first group the pyuria represents infection of kidney or bladder tissue, the urine in these cases flowing too freely to admit much bacteriological multiplication, whereas in the second group stagnation of the urine is the important factor and growth of bacteria occurs in the urine itself. As a tissue invader *Bact. coli* might be the most successful organism, whereas for growth in the poor medium afforded by stagnant urine the saprophytic organisms *Bact. aerogenes, P. vulgaris,* and *P. morgani* might have the advantage.

The results of the sensitivity tests compare with the findings of previous papers. *Bact. coli* is sensitive to sulphanilamide (Strauss and Finland, 1941; M.R.C. pamphlet on sulphonamides, 1943), while *Bact. aerogenes* (Warner, 1948), *P. vulgaris* (M.R.C. pamphlet; Stewart, 1945), and *P. morgani* (Neter and Clark, 1944) are relatively non-sensitive. *Bact. coli* and *P. vulgaris* are relatively sensitive to penicillin (Warner, 1948; Stewart, 1945; Peeney, 1947), *Bact. aerogenes* and *P. morgani* are not (Warner, 1948; Helmholz and Sung, 1944; Peeney, 1947). On the other hand, Warner found that the 12 *Bact. coli* strains tested as well as the *Bact. aerogenes* strains were insensitive to 50 mg./100 ml. of sulphathiazole.

Comparing the results of the sensitivity tests with the incidence of the various types of coliform organisms investigated, success in the treatment with sulphanilamide might be expected in the non-obstructive cases since *Bact. coli* is the commonest infecting organism, but not in the cases secondary to obstruction because coliform organisms which are not sensitive predominate in this type of infection. The use of penicillin might improve results in the cases of urinary obstruction because of its additional action on *P. vulgaris*. In the present series, however, although *P. vulgaris* was found in the urine of seventeen out of forty cases of infection secondary to obstruction, in twelve of these cases it was accompanied by *Bact. aerogenes* or *P. morgani*, organisms insensitive to penicillin. In cases of urinary obstruction there is the additional factor that while the obstruction is unrelieved there is a continuing liability to reinfection; but even if this factor is disregarded the series shows the prospects for chemotherapy by sulphanilamide and penicillin are not good.

These findings are in accord with clinical experience. Thus Brown (1946), discussing the treatment of the urinary infections of pregnancy with sulphanilamide, states the results are very satisfactory; and Donovan (1947) found that sulphonamides would not sterilize the urines of his paraplegic patients.

Streptomycin, when it becomes available, would seem to offer for the first time the possibility of obtaining a bacteriostatic concentration effective against all types of coliform bacilli found in urinary obstruction. However, the liability to reinfection on withdrawal of the drug remains, and reports emphasize the rapid development of insensitive strains. Perhaps the most useful func-

tion of streptomycin will prove to be the obtaining of a sterile field for a limited period during which a surgical operation for relief of obstruction can be carried out.

Summary

1. The incidence of different types of coliform bacilli in cases of urinary infection has been studied. A comparison has been made between types isolated from cases of primary urinary infection and urinary obstruction. Sensitivities to sulphanilamides, penicillin, and streptomycin were determined.

2. In cases of primary urinary infection *Bact. coli* was the predominant organism ; in cases secondary to urinary obstruction *Bact. aerogenes, P. vulgaris,* and *P. morgani* predominated.

3. *Bact. coli* is sensitive to sulphanilamide and penicillin in concentration obtainable in urine. *P. vulgaris* is sensitive to penicillin, but *P. morgani* and *Bact aerogenes* are sensitive to neither of these drugs. All types of coliform bacilli tested are sensitive to streptomycin.

4. Because of the high incidence of non-sensitive strains found in cases of urinary infection secondary to obstruction, no benefit from treatment with sulphonamides or penicillin is likely.

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TECHNICAL METHODS

A SIMPLIFIED PROCEDURE FOR BLOOD CELL COUNTS AND HAEMOGLOBIN DETERMINATION

BY

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Blood cell counts and haemoglobin determinations are so common in modern medical practice that even minor technical simplifications may result in significant savings of time, effort, and expense. Using standard methods the blood-counting technician to-day carries a multitude of pipettes on his rounds. These pipettes must be carefully filled to a mark with blood, and then with diluting fluid, care being taken to avoid air bubbles. Later, the pipettes must be individually cleansed, using suction apparatus.

The method described in this report requires no pipettes or filling-to-mark procedures· at the patient's bedside. It employs the principle of a calibrated capillary tube for blood sampling described by Goldfeder and her co-workers (1948) in animals. The greater simplicity of this method over the orthodox procedure suggested its use in clinical medicine. A comparative study was made ·in order to evaluate its accuracy as compared with the standard method for determination of the erythrocyte and leucocyte counts and haemoglobin concentration of the peripheral blood.

Procedure

Capillary tubes are prepared and calibrated to contain the amount of blood equal to the 0.5 mark of the Thoma blood cell pipette. This capillary tube is filled with blood obtained by finger puncture, and then diluted with a volume of diluting fluid (Hayem's solution for red cells, 2 per cent acetic acid for white, 0.1N hydrochloric acid for acid haematin formation), and the blood count or haemoglobin determination is made in the usual fashion using a Neubauer Brite-line counting-chamber and Sahli haemometer.*

Mercury is drawn into a standard Thoma white-cell pipette up to the 0.5 mark, and the measured volume of mercury is transferred to a length of capillary tubing through a wide end. Lengths of capillary tubing containing the measured amount of mercury are marked off with a diamond-point pencil, and these calibrated lengths are then separated into individual tubes. Thus, each capillary tube contains exactly the volume of blood ordinarily drawn into the white-cell pipette up to the 0.5 mark. Capillary tubes for the red-cell and haemoglobin determinations are prepared by the same technique using the corresponding pipettes. By this technique, a large supply of calibrated capillary tubes can be prepared at one time.

Red Cell Count.—Diluting fluid (Hayem's solution) is drawn into a standard red cell pipette up to the 101 mark, and then transferred to a small test tube (75 × 10 mm.). The finger-tip is punctured. A calibrated red-cell capillary tube, held in a pair of forceps, is touched to the finger-tip ·blood, and this results in immediate filling of the tube ·by. capillary action. The blood-filled capillary is then dropped into the test tube containing the diluting fluid,† after which mixing is readily accomplished by shaking. A drop of red-cell suspension is then transferred to a counting chamber by means of a glass rod· or applicator· stick, and the cells· are counted by the usual method.

White Cell Count.—The method is in every way identical with that described for the red cells, except that the corresponding diluting fluid (2 per cent acetic acid), diluting volume,‡ and calibrated white cell capillary tube ·is used.

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* The method is applicable to the making of haemoglobin determinations with a photoelectric colorimeter, acid or alkaline diluent. During the war Dr. J. Murray Steele (then commander, U.S.N.) employed a similar procedure with a photoelectric colorimeter.

† By this method the blood is diluted 1:201, as compared to 1:200 by the standard method. This introduces a negligible error of 0.5 per cent in the final red cell count.

‡ The standard white-cell pipette dilutes blood 1:20. By the method here described the dilution is 1:21, a difference of 5 per cent. which is barely significant. This small error can be corrected by using a recalibrated pipette or by withdrawing from the delivered diluting fluid the equivalent excess volume, or simply by adding 5 per cent to the final white count.

TABLE I

BLOOD COUNTS ON TWENTY-FIVE PATIENTS

Patient	Haemoglobin (g./100 ml.)				Red cells (millions/mm.³)				White cells (hundreds/mm.³)			
	Capillary		Standard		Capillary		Standard		Capillary		Standard	
1	12.0	11.5	12.0	12.0	4.4	4.3	4.1	4.7	78	76	78	70
2	12.0	11.0	11.0	11.5	3.2	3.1	4.0	2.5	168	158	132	148
3	12.5	13.0	12.5	13.0	3.2	3.5	4.2	3.9	80	86	74	86
4	12.0	12.0	12.5	12.0	3.4	3.2	4.5	3.7	70	69	70	63
5	6.5	6.5	5.5	6.0	1.3	1.3	1.5	1.2	34	32	34	32
6	12.5	13.0	12.5	12.5	3.4	3.5	4.0	3.5	114	116	100	110
7	12.0	13.0	13.0	12.0	4.5	4.6	4.7	3.5	90	80	96	82
8	10.5	11.0	11.0	10.5	3.0	2.8	3.4	3.4	106	106	98	114
9	12.0	11.5	11.5	11.5	4.6	4.4	4.6	4.7	132	120	80	118
10	14.0	14.0	14.0	13.0	3.7	3.4	3.7	3.6	84	79	142	118
11	13.0	13.0	13.5	12.5	5.2	5.0	4.8	4.3	104	94	100	84
12	14.0	14.0	14.0	14.0	4.3	4.3	4.4	4.4	104	98	90	94
13	12.5	12.0	12.5	12.5	5.2	4.9	4.8	4.5	102	100	86	78
14	13.0	14.0	14.0	13.0	4.9	4.8	5.2	5.8	68	74	70	58
15	10.5	11.0	10.5	10.5	5.4	4.9	5.1	4.7	118	130	130	120
16	14.0	14.0	13.0	13.0	5.7	4.5	4.9	4.3	100	96	104	96
17	13.5	13.0	13.5	14.0	3.8	3.8	4.0	4.1	78	92	98	76
18	13.0	13.0	13.0	12.5	4.2	4.3	3.4	3.8	88	106	92	105
19	13.5	14.0	14.0	14.0	3.3	3.4	3.5	3.7	68	76	78	72
20	12.0	12.0	12.0	12.0	3.7	3.5	4.0	4.0	82	80	82	78
21	14.0	14.0	14.5	14.0	4.2	4.3	4.2	4.2	68	64	70	68
22	12.0	12.5	12.5	13.0	3.2	3.2	4.0	3.4	78	78	76	64
23	12.0	12.0	12.0	12.5	3.0	3.2	3.1	3.0	84	84	74	74
24	13.5	14.0	13.0	13.0	3.1	3.4	3.3	3.6	178	190	188	194
25	12.5	12.0	12.0	12.0	3.7	3.7	3.6	3.7	62	66	66	70

Haemoglobin Determinations.—Dilute (0.1N) hydrochloric acid is added to the comparison tube of the Sahli haemometer in the usual manner. Using a calibrated haemoglobin capillary tube, finger-tip blood is obtained and the blood-filled capillary dropped into the comparison tube, which is then shaken to accomplish mixing. Distilled water is added until the colour matches the standard.

Data

Blood counts were made on twenty-five patients employing both the standard method as described by Todd and Sanford (1943) and the modified capillary method. Each determination was made twice by each method, totalling four complete blood counts on each patient. The results are shown in Table I. The data in Table II demonstrate that the figures obtained by paired determinations using capillary tubes for cell counts differ from each other less than the results with standard pipettes.

The statistical significance of these differences of the means of columns 3 from 4 and of column 5 from 6 have been calculated,* and yield a probability of about 2 in 100 that these differences are due to chance alone. On the other hand, the mean differences between paired determinations of haemoglobin showed the same value by both methods.

Discussion

The advantages of the capillary tube method described are especially apparent in hospitals and clinics where many blood counts are done daily. Only one set of pipettes is required for any number of complete blood counts. The capillary tubes can be calibrated readily in the laboratory,

* Comparison of the mean difference of paired red counts by the standard method to the mean difference of paired red counts by the capillary method yielded a t value of 2.15. The t value for the same comparison for white counts was 2.6. t is calculated from the form t =

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sigma_{\bar{x}_1 - \bar{x}_2}} \text{ where } \sigma_{\bar{x}_1 - \bar{x}_2} = \sqrt{\sigma_{\bar{x}_1}^2 - \sigma_{\bar{x}_2}^2} \qquad \sigma_{\bar{x}}^2 = \frac{\Sigma (x - \bar{x})^2}{N(N-1)}$$

TABLE II

TABLE OF DIFFERENCES BETWEEN PAIRED DETERMINATIONS

	Haemoglobin (g./100 mm.³)		Red blood count (million/mm.³)		White blood count (hundred/mm.³)	
	1 Standard	2 Capillary	3 Standard	4 Capillary	5 Standard	6 Capillary
1	—	0.5	0.6	0.1	8	2
2	0.5	1.0	1.5	0.1	16	10
3	0,5	0.5	0.3	0.3	12	6
4	0.5	—	0.8	0.2	7	1
5	0.5	—	0.3	—	2	2
6	—	0.5	0.5	0.1	10	2
7	1.0	1.0	1.2	0.1	14	10
8	0.5	0.5	—	0.2	16	—
9	—	0.5	0.1	0.2	38	12
10	1.0	—	0.1	0.3	24	5
11	1.0	—	0.5	0.2	16	10
12	—	—	—	—	4	6
13	—	0.5	0.3	0.3	8	2
14	1.0	1.0	0.6	0.1	12	6
15	—	0.5	0.4	0.5	10	12
16	—	—	0.6	1.2	8	4
17	0.5	0.5	0.1	—	22	14
18	0.5	—	0.4	0.1	14	18
19	—	0.5	0.2	0.1	6	8
20	—	—	—	0.2	4	2
21	0.5	—	—	0.1	2	4
22	0.5	0.5	0.6	—	12	—
23	0.5	—	0.1	0.2	—	—
24	—	0.5	0.3	0.3	6	12
25	—	0.5	0.1	—	4	4
Total	9.0	9.0	9.6	4.9	275.0	152.0
Average	0.36	0.36	0.384	0.196	11.0	6.08

and a supply of each kind can be kept available. The tubes may be re-used indefinitely after they have been placed in a detergent or in a cleaning solution, rinsed, and dried. The capillary tube method permits all the measuring of diluting fluid to be done in the laboratory at one time, away from the patient and the bedside, and simplifies the blood sampling. There is no problem of obtaining too large or too small a quantity of blood by missing the mark. The technique of capillary filling works as well on oxalated blood as on finger-tip blood. The procedure is highly economical because it eliminates the need for large numbers of blood-cell-counting pipettes. The cost of capillary tubes is negligible.

Summary

A modified method of blood cell counting and haemoglobin determination by the use of calibrated capillary tubes is described. When it was compared with the standard procedure it was found to be accurate and to give results which fluctuate less than those of the orthodox method. The new technique is economical in that it is time-saving and eliminates the need for large quantities of cell-count and haemoglobin pipettes.

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RAPID DETERMINATION OF THE SICKLE CELL TRAIT BY THE USE OF A REDUCING AGENT

BY

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Various attempts have been made to find a more satisfactory method for the determination of the sickle cell trait than the sealed wet preparation, the deficiencies of which are well known. There is need for a rapid simple technique, giving a clear-cut reading, which will serve for the rapid exclusion of sickle cell anaemia in clinical practice and also for the purpose of uniform survey work. The various techniques have been summarized by Diggs and Pettit (1940) and by Singer and Robin (1948). Because of the speed of the reaction the rapid " bacteriologic test " devised by the latter workers is an advance over previous methods, but its increased complexity is a disadvantage. For example, a living culture of *Bacillus subtilis* and an incubator are not available in the clinical side-room where a test of this nature is most wanted.

The original observation of Hahn (1928), confirmed and elaborated by Sherman (1940), established that the phenomenon of sickling only occurs in the blood of people with the sickle cell trait when the corpuscular haemoglobin is in a reduced or dissociated state, the change being accelerated by a low *p*H. Search was therefore made for an acid reducing agent which, while causing no damage to the red cell in isotonic solution, would bring about rapid dissociation of oxyhaemoglobin and so reveal sickling in a short time.

The experience of one of us in preparing haemoglobin solutions for teaching purposes suggested sodium hydrosulphite (dithionite, $Na_2S_2O_4$) as a likely substance, and this was selected for trial. A 2.1 per cent aqueous solution was found to be isotonic and to have a *p*H of 6.4. It has proved an excellent and reliable reagent for the quick demonstration of the sickling trait.

Method

Sodium hydrosulphite is a powerful reducing agent which decomposes in moist air to a mixture of sodium thiosulphate and sodium sulphite. A solution, therefore, will not keep indefinitely. For the preparation of the solution distilled water which has been freshly boiled and allowed to cool is used. The 2.1 per cent solution is made once a week, and kept in small tubes with a covering seal of liquid paraffin. Once the paraffin seal is removed, the tube should be discarded at the end of the day.

Technique and Reading of Test.—A drop of the isotonic sodium hydrosulphite solution is taken up in a finely drawn Pasteur pipette, and about one-sixth of this quantity of blood (from either a skin prick or venepuncture) is drawn up in apposition to the column of fluid. The whole is then expelled on to a clean slide and rapidly mixed. The slide is inverted on to a coverslip and examined under the 1/6 objective. It is not necessary to seal the coverslip with vaseline. The same pipette may be rinsed in normal saline and used for any number of patients in rapid succession.

If the sickle cell trait is present, sickling will occur in from 3 to 15 minutes, and rapidly become definite and generalized. It tends to appear earliest in cells near the edge of the coverslip, possibly due to the effect of surface tension on the rate of reaction. If there is no sickling after 20 minutes the blood can be pronounced negative for the sickle cell trait. Sickling never occurs in normal blood, though the red cells may undergo crenation and haemolyse after half an hour.

Comparison with Sealed Wet Preparation.—One hundred African schoolboys were examined by both the sealed wet preparation, read at 48 hours, and the sodium hydrosulphite technique. There was complete agreement, both as to positives and negatives, between the two methods. (The numerical findings form part of a survey which will be published separately.) The sodium hydrosulphite technique always gave clear-cut results within 20 minutes, whereas with the sealed wet preparation the reading was sometimes still doubtful after 24 hours.

Summary

A technique is described for routine determination of the sickling trait, employing a reducing agent to produce rapid dissociation of oxyhaemoglobin. A drop of a freshly prepared 2.1 per cent aqueous solution of sodium hydrosulphite is mixed

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with about one-sixth of its volume of blood. The suspension of red cells is observed under a cover-slip for up to 20 minutes. No seal is necessary. In speed, simplicity, and reliability this procedure is considered superior to other methods in use at the present time.

Our thanks are due to Dr. W. D. Raymond, Government chemist, Tanganyika, for his assistance and advice in this investigation, and to the Director of Medical Services, Tanganyika, for permission to publish.

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Shortly after submitting this paper for publication, the work of Thomas and Stetson (*Johns Hopk. Hosp. Bull.*, 1948, **83**, 176) came to our notice. Of sulphydryl compounds which they tried, a saturated solution of hydrogen sulphide was found to be the most reliable and rapidly acting agent for the demonstration of sickling. While there is probably little to choose between the two reagents in their efficiency as indicators of sickling, sodium hydrosulphite has practical advantages as to odour, stability, and preparation of the solution.

THE PHOTOCHEMICAL PRODUCTION OF GOLD SOLS USING ARTIFICIAL LIGHT

BY

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From the Department of Chemical Pathology, University of Leeds

Twenty years ago (Fowweather, 1928) a method for the preparation of gold sols for the Lange test on cerebrospinal fluids was published in which gold potassium bromide is reduced by potassium oxalate under the action of daylight. Temperature and light intensity were found to be complementary to some extent; thus it was found advisable to carry out the reaction at a somewhat raised temperature in winter, when daylight intensity is comparatively low. Hoess (1931) advocated the use of a " vitalux " lamp, and Herrmann (1935) measured the light intensity by a light meter and adjusted the temperature to the intensity of light. The modifications were not entirely satisfactory, and the search for a satisfactory light source which should be reasonably constant during the course of a preparation continued; two methods were successfully developed during 1940, and one or other has been in use here ever since. The first uses an ultra-violet lamp and the second a high-power electric lamp of conventional pattern.

Method I

The lamp used is a Hanovia mercury-vapour lamp of the type used for therapeutic ultra-violet irradiation.

400 ml. of freshly distilled water* is placed in a 500-ml. conical flask having a ground-glass stopper, and the temperature of the water is adjusted to 25° C. 4 ml. of a freshly prepared 1 per cent solution of potassium oxalate (" AnalaR ") is added, followed by 6 ml. of a 1 per cent solution of gold potassium bromide, and the contents of the flask are thoroughly mixed. (The latter solution need not be freshly prepared, provided it has been kept in the dark in a tightly stoppered bottle, but it is inadvisable to use solutions more than a month old.)

The flask is placed on a bench and the height of the lamp is adjusted so that the mercury tube is

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* It is not necessary to use doubly distilled water; the important point is that water should be *freshly* distilled (Herrmann, 1932).

opposite the middle of the flask, and the distance of the tube from the centre of the flask is 11 in. It is probable that the most satisfactory distance between lamp and flask varies with the lamp used, so that the correct distance for any particular lamp should be determined by experiment. Immediately behind the flask is an upright three-piece wooden screen with short centre-piece parallel to the mercury tube, and longer wings fixed at each end at an angle of 135°. This is painted white, and its purpose is to reflect the rays falling on it from the lamp inwards to the flask. As irradiation of the flask proceeds, the brown colour of the solution fades, and after about 12 minutes the solution is colourless. Then red streaks appear and spread through the solution, and soon the typical gold sol is fully developed, the total irradiation period being about 18 minutes. The flask is then placed in diffuse daylight for 3 or 4 hours, after which it is stored in the dark for 2 or 3 weeks before being used, following the recommendation of Herrmann (1932).

Method II

The lamp is a standard Osram 1,000-watt lamp with clear, uncoloured glass. It is used with a white reflector behind it, as well as one behind the flask. The most satisfactory distance between the centre of the lamp and that of the flask was found to be 13 in., and the period of irradiation about 25 minutes. All other procedures are the same as those employed with the ultra-violet lamp.

Discussion

The sols prepared by the second method are more cloudy than those prepared by the first, and on the whole the first method is preferred. The second was used successfully over a long period when the Hanovia lamp was not available. The sol has recently been successfully prepared in 1,000 ml. quantities by the first method (using 1 ml. of oxalate solution and 1.5 ml. of bromide solution for each 100 ml. of water).

Difficulties caused by undue stability of sols prepared by these methods have not been met, though there is some evidence that these would occur if sols were still employed more than about three weeks after they have first been brought into use. It is advisable, therefore, to prepare only such quantities as will be used within this period. Occasionally sols are produced that are too sensitive and show a change with normal cerebrospinal fluid, usually of one or two degrees, in the second and third tubes of the Lange test range. Such sols are produced more frequently by the second method than by the first—an additional reason for preferring the latter. This sensitivity can, however, be removed by heating the sol to about 90° C. and then allowing it to cool. Hence, whenever a new batch is to be brought into use a Lange test on a normal cerebrospinal fluid is made, and this treatment is applied if undue sensitivity is shown.

Sols prepared by the methods described are equally suitable for cerebrospinal fluid or serum colloidal gold tests.

If a transparent silica flask is used instead of a glass flask, the period of irradiation is shortened considerably in the first method, but is not appreciably altered in the second. Silica transmits ultra-violet light much better than glass, and it would appear, therefore, that in the first method the ultra-violet light plays a considerable part in the reaction, while in the second it is only visible light which is present and is effective. Hence the range of light frequencies capable of bringing about the reduction of the gold salt by potassium oxalate is not a very narrow one, since it includes part of the visible as well as the ultra-violet portion of the spectrum.

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ORTHOTOLIDINE HYDROCHLORIDE TEST FOR BLOOD IN URINE

BY

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In 1943 the author described a test for blood in urine in which small squares of filter paper impregnated with *o*-tolidine were used. The papers, however, deteriorated within a few months. This disadvantage has now been overcome by using solid *o*-tolidine hydrochloride instead of the papers.

Reagents

o-Tolidine hydrochloride. Acid-peroxide solution. Equal volumes of glacial acetic acid and 3 per cent hydrogen peroxide (freshly diluted from 30 per cent). The mixture is allowed to stand for twenty-four hours before using. The solution should be freshly prepared every three months.

Procedure

A small knife-point (about 3 mg.) of solid *o*-tolidine hydrochloride is placed on a clean white porcelain tile. One drop of urine is added and thoroughly mixed with the solid by stirring with a glass rod. One drop of acid-peroxide solution is added.

Reactions

Negative reaction.—A light brown colour slowly develops. Occasionally one or two large blue dots appear. These must be disregarded.

Positive reactions.—(*a*) Numerous small greenish-blue dots develop within a few seconds. Sometimes the dots are drawn out into greenish-blue streaks. The dots or streaks persist for periods varying from ten to thirty seconds to several minutes according to the amount of blood present. They then fade and disappear and a light brown colour develops. Reaction (*a*) is the typical reaction for occult blood (red cells) in urine.

(*b*) When a large amount of blood is present, such as 1,000 red cells per c.mm., a dense, dark blue colour appears immediately. The dark blue mass is surrounded by yellowish-green areas. The colour may persist for half an hour or longer.

(*c*) If free haemoglobin is present a diffuse greenish-blue to dark blue colour appears which persists for a varying length of time and then fades.

False positive reactions.—Bromides and iodides in the urine give positive reactions. These will obscure the reactions for blood in a urine which contains both blood and bromides or iodides. Bromides give a diffuse greenish to greenish-blue colour which develops slowly and then fades. The reactions given by iodides are similar to the blood reactions (*b*) and (*c*) described above. Small amounts of iodides give a greenish-blue colour which also quickly fades to a light brown colour.

A true positive reaction due to blood can be distinguished from a false positive reaction due to bromides or iodides as follows.

A few ml. of urine are boiled for about one minute and then cooled, and the test is repeated. If blood is present the second test will be negative or the colour will be reduced in intensity according to the amount of blood originally present. Urines which contain as many as 1,000 red cells per c.mm. show a decreased reaction after boiling. Iodides and bromides, even when present in small amounts, are unaffected by boiling. Therefore if a positive reaction is decreased or is negative when the test is repeated after boiling, it can only be due to the presence of blood.

Pus gives a negative reaction unless it is present in such large amounts as to cause an obvious turbidity.

It has been reported that an excess of ascorbic acid in urine inhibits the benzidine blood test (Kohn and Watrous, 1938) and also the *o*-tolidine test performed on the centrifuged deposit (Barach and Pennock, 1940). The author has found that it also inhibits the *o*-tolidine hydrochloride test described above, and as a result small amounts of blood will give a negative reaction in the presence of an excess of ascorbic acid.

Sensitivity of the Test

In the experiments for determining the sensitivity of the test it was assumed that the blood

used contained 5 million red cells per c.mm. and 15 g. haemoglobin per 100 ml. This is a sufficiently close approximation for the purpose of assessing the sensitivity of a qualitative test.

1. Haematuria.—0.1 ml. of blood was pipetted into about 90 ml. of 0.9 per cent sodium chloride and made up to 100 ml. with saline. Serial dilutions were then prepared with normal urine and the *o*-tolidine hydrochloride test performed on the urines containing various concentrations of red cells.

It was found that a concentration of 1 red cell per c.mm. (=a dilution of 1 in 5 million) gave a negative result. A urine containing 2 red cells per c.mm. gave a slight but definite positive reaction ; a concentration of 5 red cells per c.mm. (=a dilution of 1 in one million) gave a comparatively strong positive reaction. According to Stone and Burke (1934), who applied an *o*-tolidine test to centrifuged deposits of urine, the persistent excretion of more than 1 red cell per c.mm. of urine is of clinical significance and merits further investigation. They found that red cells are not ordinarily detected with the microscope until their number exceeds 5 per c.mm. of urine. The *o*-tolidine hydrochloride test is therefore more sensitive than the microscopical test and its sensitivity is such that a slight but definite positive reaction is of pathological significance.

2. Haemoglobinuria.—0.1 ml. of blood was pipetted into about 90 ml. of distilled water and made up to 100 ml. with distilled water. Serial dilutions were prepared with normal urine and the various dilutions tested.

The smallest amount of free haemoglobin detectable in urine was found to be 150 μg. per 100 ml. (the equivalent of a dilution of 1 in 100,000 or 50 red cells per c.mm.). On spectroscopic examination the smallest amount of haemoglobin detectable was 2.4 mg. per 100 ml. (equivalent of 800 red cells per c.mm.). The examination was made with a direct-vision spectroscope through a layer of urine two inches thick.

The *o*-tolidine hydrochloride test is, thus, more sensitive than the microscopical test for red cells and about sixteen times more sensitive than the spectroscopic test for free haemoglobin. It is about twenty-five times more sensitive for red cells in suspension than for free haemoglobin in solution.

Summary

1. A simple chemical test for blood in urine is described. Solid *o*-tolidine hydrochloride is used for the test, which is performed with one drop of urine and one drop of an acetic acid—hydrogen peroxide mixture.

2. After boiling, urines containing blood give a decreased or negative reaction. Iodides and bromides, which also give positive reactions, are unaffected by boiling.

3. Pus gives a negative reaction unless it is present in such large amounts as to cause an obvious turbidity.

4. Excess ascorbic acid decreases the sensitivity of the test.

5. The test is more sensitive than the microscopical test for red cells and about sixteen times more sensitive than the spectroscopic test for free haemoglobin. The smallest number of red cells detectable is 2 per c.mm. and the smallest amount of free haemoglobin detectable is 150 μg. per 100 ml.

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THE MASS STAINING OF PARAFFIN SECTIONS BEFORE THE REMOVAL OF WAX

BY

C. F. A. CULLING

From the Westminster School of Medicine

The problem of producing large numbers of sections for teaching purposes and staining them by haematoxylin and eosin has become acute in the post-war years, due to the shortage both of trained technicians and of reagents, especially ethyl alcohol. Methods previously used have suffered from the disadvantages of being too long, or wasteful of reagents, and it was with these factors in mind that the following method was evolved.

Robb-Smith (1937) showed that free paraffin sections could be impregnated with silver. Mayer also showed that methyl violet would stain amyloid in paraffin sections that had not been de-waxed. This principle has been adapted for the mass staining of paraffin sections before the removal of wax.

Apparatus

An M.S.E. rotary-type microtome is used in this department as it is thought to give better ribbons of sections. Half of a 7-in. Petri dish or a developing-dish of similar size has been found sufficient for 60 sections of average size (2 cm. × 1 cm.). Four wash-bottles with rubber and glass attachments may be prepared from empty wide-mouthed bottles, rubber-and-glass tubing, and rubber bungs. A glass filter pump is at present used, positive pressure being obtained by blowing. Dissecting needles and staining solutions are also needed.

Method

Some sixty to seventy sections are cut in the usual manner. The use of ice on the surface of the paraffin block before and during cutting gives flatter sections.

The dish is half-filled with water at a temperature of approximately 45° C. when using 54° C.M.P. wax (see Appendix).

Ribbons are cut to a convenient length to give half-inch clearance each end when flattened in the dish. The method of removal and replacement of solutions follows the same pattern throughout and is as follows.

The glass tube connected to the wash-bottle (see Appendix, 2) is passed down the side of the dish to the bottom, and negative pressure is applied. While the surface of the water is falling, the ribbons are kept free of the side of the dish by needles; otherwise they become attached. If this should happen, they are easily freed by turning off the filter-pump and tilting the dish gently towards the attached section. The last few millilitres are removed by tilting the dish towards the suction tube. This is done slowly, as any rapid movement will cause telescoping of sections. Pressure is reduced towards the end of this operation.

Solutions are run in by a reverse procedure, positive pressure being applied to the wash-bottle, either with a pump or by blowing to start the stream, when the remainder will siphon over if the bottle is lifted. Only enough solution to float the sections is required.

The following solutions and times have been found to give the best results with haematoxylin and eosin:

1. Tap water at 45° C. to flatten sections. Heated distilled water may be substituted for this, as it combines stages 1 and 2, but time is gained by this procedure only if hot water is not readily available.

2. Distilled water—2 minutes.

3. Haematoxylin—40 minutes (see Appendix, 3).

4. Distilled water—2 minutes.

5. Acid alcohol (1 per cent in 95 per cent alcohol) —1 minute (see Appendix, 4).

6. Ammonia water (0.1 per cent in distilled water) —until sections are blue.

7. Tap water—2 minutes.

8. Eosin (1 per cent in distilled water)—20 minutes.

9. Tap water (cold).

Sections are left as short a time as possible on tap water after stage 8, as this removes the eosin. The ribbons are now separated into sections. This is easily effected by a gentle stabbing motion with the point of the needle at the intersections.

Individual sections are now floated on to clean slides in the usual manner, and are allowed to drain by standing upright on filter paper. Blotting firmly at this stage is both expeditious and gives a flatter section.

When the sections are dry they are gently heated, placed in xylol to remove wax, and mounted in Canada balsam.

Conclusions

The results obtained with this method compare favourably with methods previously used in this department. As early sections were under-stained with eosin, it was necessary to increase the staining time and to float section on to slides as soon as possible. There seems to be no reason why other staining techniques should not be adapted for use with this method, especially those in which precipitation of stain occurs, or where one of the reagents causes the section to float off the slide.

Summary

A method is given for which the apparatus used is readily available in almost all laboratories.

A set of fifty sections may be cut and stained by one technician in approximately two and a half hours, during which time there are quite long intervals when other work may be performed.

The use of alcohol for dehydration is obviated. The handling of single slides, a time-consuming operation, is cut to a minimum.

REFERENCE

Robb-Smith, A. H. T. (1937). *J. Path. Bact.*, **45**, 312.

Appendix

1. Air bubbles in fluid are to be avoided if possible, and should be removed from warm water by running fingers round the inside of the dish before floating on sections. Bubbles that are formed subsequently do not appear to cause uneven staining.

2. If a glass filter-pump is being used a wash-bottle is not needed when removing tap water or distilled water, as these are obviously used fresh each time. If a pump of the Hyvac type is used a wash-bottle is, of course, essential.

3. Harris's haematoxylin has been used, staining with which compares favourably with Ehrlich's, and it has the advantage of being easily and rapidly prepared. Since it is ripened with mercuric oxide, it is immediately ready for use. Glacial acetic acid (5 per cent) is added, to give more precise nuclear staining, and to neutralize any salts carried over from the tap water.

4. Differentiation may be controlled microscopically in the usual manner by floating a single section on to a clean slide. The acid alcohol is, of course, omitted if the progressive method of staining is preferred.

AN IMPROVED SWAB FOR THE DETECTION OF THREADWORM OVA

BY

J. A. BOYCOTT

The following device has been found to have certain advantages over the N.I.H. swabs* in the detection of the ova of threadworms (Oxyuris vermicularis).

It consists of: (1) a wooden tongue depressor 6 in. by ¼ in. ; (2) a strip of cellophane approximately 3 in. by 1 in., the size of a microscope slide, doubled over the end of the depressor and held in place by several turns of a rubber band ; (3) a paper envelope: a " pay envelope," 2 in. by 4 in., is suitable.

Swabbing is most effective if done immediately the patient wakes in the morning. The swab should be rubbed along the peri-anal folds using pressure just short of causing pain. It is then replaced in the envelope, on which it is convenient to write the patient's name. In the laboratory the cellophane is removed, opened out, and placed on a slide ; a drop of Canada balsam between slide and cellophane helps to keep the surface flat. Examination should be made under the ⅔-in. objective and may be concentrated in the areas showing epithelial and faecal debris.

The single crease in the cellophane, the strength, and ease of packing have been found to be improvements on the N.I.H. swab.

* Hall, M.C. (1937). *Amer. J. trop. Med.* **17**, 445.

REVIEWS

An Atlas of Bone-Marrow Pathology. By M. C. G. Israëls, M.Sc., M.D., M.R.C.P. William Heinemann Medical Books, Ltd. Pp. 50. 12 plates. Price 30s. net.

Bone Marrow Biopsy. By S. J. Leitner, M.D. Translated by C. J. C. Britton, M.B., Ch.B., D.P.H., and E. Neumark, M.B., B.S.(Lond.), M.R.C.S., L.R.C.P. J. & A. Churchill, Ltd. Pp. 433. 7 plates. 194 text-figures. Price 42s. net.

Bone marrow biopsy has become one of the accepted routine investigations carried out in the wards and often in the out-patient departments in most hospitals. It is used in the diagnosis and control follow-up of many conditions, particularly, of course, in haematological disorders.

There has long been felt the need for an authoritative book and atlas, and there was in particular an opportunity for a prominent haematologist to give a clear lead on the nomenclature and classification of the cells seen in smears and sections from bone marrow biopsy. This necessity has been intensified by the recent suggestions for an agreed international terminology and even more, as Israëls recently said, for a clear-cut description of the cells which we call by the new or old names. There is, for example, no international agreement on the term "megaloblast." Israëls, therefore, in the introduction to his book proclaims his intention "to set out an authoritative, accurately illustrated account of the bone marrow in health and disease, for the guidance of physicians and pathologists." This intention is unfortunately not fulfilled, as indeed it could never have been, in a book of only 50 pages and 12 plates. It does, however, provide a non-controversial introduction to the subject, and as such will be read and appreciated by physicians and by students who will thereby be enabled the more easily to understand the pathologists' reports. The colour drawings are excellent and do give a clear and firm description of the cells described. A minor source of annoyance could be remedied in future editions if the interleaved pages with contour sketches of the plates 8–12 were omitted. It is very difficult to keep a picture of a microscopic field and pick out cells for identification as is intended in these plates, when the process involves unnecessary procedures of number identification on an intermediate sheet and the key on yet another page. The plates 1–7 are so much easier to follow that the tracings should be regarded as superfluous.

For the pathologist who requires an authoritative reference book Israëls will be quite inadequate, and so it was with even greater expectations that the book by Leitner was received. This book has essentially a clinical outlook and throughout there are numerous illustrative case histories which are valuable. The translators were faced with two problems: one to provide an easy translation, which they have in the main achieved, although the rather stilted style in places is evidence of their difficulties; and the other to attract British readers with a book which had to be made not too obviously Continental in outlook. The senior translator admits his previous intention to undertake a similar book on his own account, and he has compromised by *incorporating many British and American references to avoid another publication*. This volume will almost certainly become a standard textbook in Great Britain and it can be assumed that the licence that the translators have been allowed will continue and even be extended so that later editions will contain a greater proportion of works with which we are more familiar in this country. On the whole the photographs and plates are good, but it is most disturbing to find such a variety of magnifications used in the photomicrographs. Magnifications of 50, 100, 120, 150, 500, 600, 650, 750, 1,000, 1,050, and 1,400 are used, and frequently several on one page. In fact, the only magnifications which are really satisfactory are the 1,000 and the 1,400, and many of these are really excellent, whereas some of the smaller ones—e.g., on pages 359, 115, and 159—are merely a collection of black cells without meaning. The photomicrographs should be carefully scrutinized before the next issue.

The other major criticism is the number of references. Although this book can well claim to be a reference book, it is really quite unreadable in parts because of the intrusion, line after line, of meaningless names and dates. This can best be appreciated by the fact that throughout the chapter on erythropoeisis there are 1,180 references, which also occupy 15 pages at the end of the chapter. It can be confidently assumed that the book would not suffer if this number were to be reduced to a quarter.

In our last issue we published an admirable review by Dacie and White on bone marrow biopsy, with special reference to erythropoeisis. This review was widely appreciated, and, with the two books here reviewed, should provide a very solid basis for the better understanding of the bone marrow in health and disease.

A. G. SIGNY.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology.; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Streptomycin in Treatment of Tuberculosis and Mixed Infections of the Genito-urinary Organs. REDEWILL, F. H., and POTTER, J. E. (1948). *Urol. cutan. Rev.*, 52, 259.

The synergic action of streptomycin, penicillin, and sulphonamides allows smaller doses of each drug to be used without loss of efficiency and with less toxic effects. Streptomycin-resistant strains of a bacillus can develop only when the concentration of this drug is inadequate to depress bacterial virulence sufficiently to allow tissue healing to occur. Once healing has begun even small doses of the antibiotic will prevent resistant strains from developing. There is no difference in the virulence of streptomycin-sensitive and streptomycin-resistant strains of the same organism. The dose advocated by the authors is small—0.8 to 1 g. daily by two separate injections. The treatment of 22 cases of genito-urinary tuberculosis is reported; 2 did not improve, 6 improved, and 14 were apparently cured. Of these, 5 were cases of primary tuberculous cystitis, 7 of tuberculous nephritis in the remaining kidney, 8 of tuberculous nephritis with complications, and 2 of tuberculous prostatitis. In 120 cases of acute and chronic non-tuberculous urological infection also treated, the highest percentage of cured and improved cases yet reported was obtained by: (*a*) careful assay of the case before treatment; (*b*) rejection of cases in which streptomycin-resistant bacteria were likely to develop or the *p*H of the urine was low; and (*c*) use of a combination of streptomycin, penicillin, and "promizole." *J. E. Semple.*

Observations on Streptomycin in Tuberculosis. IV. Importance of the Cerebrospinal Fluid Picture in Tuberculous Meningitis Treated with Streptomycin. (Osservazioni sulla streptomicina nella infezione tubercolare. IV. Importanza del quadro liquorale nelle meningiti t.b.c. trattate con streptomicina.) MARTON, L. (1947). *Clin. pediat., Bologna,* 29, 716.

The author discusses the character and components of cerebrospinal fluid in 40 cases of tuberculous meningitis, treated with streptomycin. In assessing the prognosis the author finds the level of glucose in the cerebrospinal fluid to be the most helpful observation. When this is below normal, treatment should not be stopped. He gives details of the findings in 8 cases. In some, after cure cellular and protein changes persist. The author suggests that these changes are due to structural changes in the choroid plexus, the result of the antecedent disease process. *J. Maclean Smith.*

Pulmonary Tuberculosis Treated with *p*-Aminosalicylic Acid. Early Results in 6 Cases. ERDEI, A., and SNELL, W. E. (1948). *Lancet,* 1, 791.

The authors gave *para*-aminosalicylic acid (P.A.S.) by mouth to 5 patients for 60 days and to 1 patient for 4 weeks. Each patient received 12 g. daily in divided doses 3-hourly, 1 night dose being omitted. All the patients were men, and most had acute disease with toxaemia. The authors noted considerable improvement in the general condition and a lessening of the toxaemia (often within 3 days). No toxic reactions were observed. A blood level of 2 to 5 mg. per 100 ml. was attained, and the concentration in the urine was high (500 mg. per 100 ml.). The authors believe that P.A.S. has a direct bacteriostatic effect upon the bacilli, an antipyretic action, and a "direct pharmacological action on the host." They consider a daily dose of 12 g. is too low and recommend increasing it to 20 g. Vitamin-B complex should be given as well. *N. Lloyd Rusby.*

Examination for Tubercle Bacilli by Gastric Lavage and by Laryngeal Swab: A Comparative Study. HOUNSLOW, A. G., and USHER, G. (1948). *Tubercle,* 29, 25.

This study is based upon 193 patients at various stages of treatment for pulmonary tuberculosis, all of whom were sputum-free or produced scanty mucoid expectoration negative for tubercle bacilli by smear and culture examination. The results suggest that when a single tube is inoculated a single gastric lavage is more likely to give a positive result than a single laryngeal swab, but three swabs give much more precise results than does a single gastric lavage; if, however, three tubes are inoculated a single gastric lavage is superior to three swabs.

Penicillin in the Cerebrospinal Fluid Following Parenteral Penicillin. BOGER, W. P., BAKER, R. B., and WILSON, W. W. (1948). *Proc. Soc. exp. Biol., N.Y.,* 68, 101.

Twenty-six patients suffering from syphilis of the central nervous system (paresis) were treated with 100,000 units of penicillin intramuscularly every 3 hours for 3 days, followed by the same dose of penicillin together with 3 g. of caronamide (4'-carboxyphenylmethane sulphonanilide) orally every 3 hours for a further 5 days. Caronamide was given since it inhibits excretion of penicillin by the renal tubules, yielding higher concentrations of penicillin in the plasma. After the 5 days of the penicillin therapy alone, in 15 patients the levels in the cerebrospinal fluid ranged from 0.019 to

0.052 unit per ml. At the end of the further period of 5 days (during which caronamide was given as well), in 20 patients concentrations of penicillin in the cerebro-spinal fluid ranged from 0.026 to 2.5 units per ml. The levels obtained were considered to be therapeutically significant since a concentration of 0.03 unit per ml. is usually sufficient to inhibit the growth of organisms such as streptococci, meningococci, and staphylococci. A lengthy discussion is given on the penetration of penicillin through the blood-brain barrier. *R. Wien.*

Oral Penicillin in the Treatment of Various Bacterial Infections. ROBINSON, J. A., HIRSH, H. L., and DOWLING, H. F. (1948). *Amer. J. Med.*, 4, 716.

Penicillin tablets (sodium salt) buffered with calcium carbonate were administered orally in the treatment of various bacterial infections (strength of each tablet not given). In all infections, except subacute bacterial endocarditis, in which oral penicillin is not recommended because of the seriousness of the disease, the results were comparable with those obtained with parenteral penicillin. The total oral dose of penicillin was 5 times higher than the total intramuscular dose for the same infection. Toxic effects were less often observed with oral therapy.

Sycosis Barbae. Serological Types of *Staphylococcus pyogenes* in Nose and Skin and Results of Penicillin Treatment. HOBBS, B. C., CARRUTHERS, H. L., and GOUGH, J. (1947). *Lancet*, 2, 572.

Staph. pyogenes was isolated from the facial lesions of each of 23 patients. Of 19 in whom the strains were typed, 17 had the same type of staphylococcus in the nose as in the skin; 16 of these showed one type only and 1 had two. Corresponding organisms were isolated from the throat in 2 cases and from the eyes of 2 patients with blepharitis. Nine different types were isolated from the lesions: IIIc four times; I, Ib, and C.K.5 three times; 1636 twice; and Ia, IIIa, IIIb, and III/5 once each. Treatment consisted of applying cream containing 500 units of penicillin per g. in 25 or 30% neutral "Lanette wax SX" in water to the face night and morning, and inserting this cream into the nose with a throat swab and inhaling vigorously. Clinical improvement was usually immediate, and cases in which there was relapse usually responded to a second course of treatment. Relapses are due either to inactivation of the penicillin cream or to reinfection with the same or different strains. *H. R. Vickers.*

Histopathological Findings in General Paresis After Penicillin Treatment: Report on 4 Cases. SMITH, R. H. F., and DE MORLAIS, V. (1948). *J. ment. Sci.*, 94, 70.

No previous reports on the neuropathological findings in paresis after penicillin therapy have appeared. The dosage in the 4 cases reported here ranged from 2,400,000 to 4,800,000 units. In 2 of the cases there was clinical as well as serological improvement; the 2 cases which did not improve were of the juvenile type of general paresis, 1 of them a Lissauer type. Histological examination of the brain of the 2 patients who benefited revealed

less intense inflammatory changes than in untreated cases. The iron reaction was positive in all 4 patients, but was less pronounced in the 2 who improved. No spirochaetes were found in any of the brains. These findings resembled those obtained at comparable times after malaria treatment. The possibility that penicillin may produce an initial exacerbation of the process is suggested by the 2 unimproved cases. From the results it would appear that penicillin alone can influence the pathological progress of general paresis. *E. W. Anderson.*

Fatal Toxic Encephalopathy Apparently Caused by Streptomycin. HUNNICUTT, T., GRAF, W. J., HAMBURGER, M., FERRIS, E. B., and SCHEINKER, I. K. (1948). *J. Amer. med. Ass.*, 137, 599.

Streptomycin was given to a diabetic woman, aged 25, for staphylococcal infection producing active pyelonephritis and multiple skin abscesses. Symptoms of encephalopathy appeared on the fifth day of the treatment after a total dose of 15.6 g. of streptomycin had been given. There was disorientation with coarse involuntary muscular movements leading to generalized convulsions and accompanied by hyperpyrexia, tachycardia, and hypertension. Three days later the patient died in coma, with Cheyne-Stokes breathing. Uraemia, diabetic ketosis, and insulin shock were excluded by laboratory findings. Cerebrospinal fluid examined 24 hours after the drug was discontinued contained the drug in half the concentration present in the blood at that time.

Post-mortem changes in the pancreas and the kidneys were consistent with the diagnosis of diabetes mellitus and staphylococcal infection. Significant changes, similar to those seen in brain injury and other toxic and arsphenamine encephalopathies, were observed in the brain and were confined to cortical white matter, basal ganglia, hypothalamus, and medulla. Because of central vasoparalysis there was stasis in small veins and capillaries with distension and some necrosis of the vessel wall. These vascular changes in turn led to ischaemic degeneration, rarefaction, and necrosis of the nervous tissue proper. No changes were seen in the cranial nerves. The leptomeninges were congested. In the absence of affection of the cranial nerves, especially the eighth, the brain changes are believed to be due to direct action of streptomycin, and not to impurities, caused by high serum concentration. The high serum level was probably due to defective renal function, which in the authors' opinion should be adequate if streptomycin is to be given. *S. Karani.*

Studies on 3,4-Dimethyl-5-Sulfanilamido-Isoxazole (Nu-445) in Humans. SARNOFF, S. J. (1948). *Proc. Soc. exp. Biol., N.Y.*, 68, 23.

In 6 patients, each given a single dose of 3 or 4 g. orally, a blood level of 12 to 16 mg. (total) per 100 ml. was maintained from 2 to 8 hours. In 2 patients, after the oral administration of 4 g., there was only a low level of about 1 mg. per 100 ml. in the cerebrospinal fluid, but in 1 patient with meningococcal meningitis a concentration of 5 mg. per 100 ml. (total) was obtained. In a series of 37 patients the usual mild toxic sulphonamide reactions were observed, but there was no crystalluria. *R. Wien.*

Restoration of Diphtheria Immunity without Injections (Bousfield's Method). MASUCCI, P., GOLD, H., and DEFALCO, R. J. (1948). *J. Pediat.*, 32, 35.

This investigation in a series of 37 subjects confirms Bousfield's findings that diphtheria toxoid can pass across the mucous membranes of the upper alimentary tract. The amount absorbed was only sufficient to act as a "booster" dose, and primary immunization by this method does not appear feasible. The toxoid, 100 Lf units, was incorporated in a compressed tablet and three tablets were given daily for 7 days. The tablets were kept under the tongue and sucked slowly. No inconveniences or reactions were observed. The large total dosage required may restrict the practical application of this method to adults, in whom severe constitutional disturbances after a parenteral injection of diphtheria toxoid are not uncommon. *H. Herlinger.*

Combined Active and Passive Immunization Against Diphtheria. DOWNIE, A. W., GLENNY, A. T., PARISH, H. J., SPOONER, E. T. C., VOLLUM, R. L., and WILSON, G. S. (1948). *J. Hyg., Camb.*, 46, 34.

In 1941 Downie *et al.* (*Brit. med. J.*, 1941, 2, 717) concluded that a combined technique as compared with simple active immunization led to some delay, and a slight inhibition, in the formation of antitoxin by the individual. The final degree of immunity did not, however, fall far short of that resulting from active immunization alone (a Schick-conversion rate of 90.9% as against 98%).

The authors now suggest that in practice the inhibitory effect of the 500 units of antitoxin might be reduced by further increasing both doses of APT to 0.5 ml. and at the same time lengthening the interval between the injections to 6 weeks. *H. J. Bensted.*

An Outbreak of Infantile Gastro-enteritis in Aberdeen. The Association of a Special Type of *Bact. coli* with the Infection. GILES, C., and SANGSTER, G. (1948). *J. Hyg., Camb.*, 46, 1.

The authors accept the classification of infantile diarrhoea into (1) non-infective, (2) secondary or symptomatic, (3) primary or infective (*a*) with non-causative agent and (*b*) of unknown aetiology; and they investigated 159 cases of infantile diarrhoea admitted to Aberdeen City Hospital in the first 5 months of 1947. They grouped 93 cases as belonging to the primary infective group but could isolate a known pathogen *Shigella dysenteriae* (Sonne) from only one: the remaining 92 (among which there were 52 deaths, a fatality of 56.5%) were associated with a serologically homogeneous strain of *Bact. coli*, the biochemical and antigenic properties of which are described. The organism was recovered from over 90% of cases of primary gastro-enteritis, 34% of doubtful cases and secondary diarrhoea, and less than 5% of healthy controls; efforts to demonstrate the pathogenicity of the organism or the presence of a concomitant virus were unsuccessful.

The Treatment of Pertussis and Pneumonia Complicating Pertussis. The Role of Hyperimmune Gamma Globulin and Sulfadiazine. BRAINERD, H. (1948). *J. Pediat.*, 32, 30.

The author treated 26 infants suffering from pertussis with hyperimmune gamma globulin (agglutinating titre of at least 1 in 15,000), injecting 2.5 ml. intramuscularly on each of 4 successive days. In addition sulphadiazine in doses of 130 mg. per kilo a day was given to another 26 infants in whom pertussis was complicated by pneumonia. The mortality in this latter group was only 7.7%. The response to treatment was often dramatic. The best results were obtained if treatment began in the first 7 days of illness. *H. Herlinger.*

The Relationship Between the Antipoliomyelitic Properties of Human Nasopharyngeal Secretions and Blood Serums. BELL, E. J. (1948). *Amer. J. Hyg.*, 47, 351.

The secretions of 32 to 68 "normal" adults neutralized the Lansing strain of poliomyelitis virus. The serum of 51 of 68 persons also neutralized and that of 17 persons failed in this test. Of 32 persons with secretions that neutralized the virus all but 2 showed serum antibody also. Of the 51 whose serum neutralized, fewer than two-thirds had positive secretions. It appeared that above a certain level antibody "spilled over" from the serum.

Massive Colony Formation of *Bacterium friedländeri* in the Liver in Agranulocytosis. DAVIDSON, J. I. (1948). *J. Path. Bact.*, 60, 51.

A common cause of death in agranulocytosis is bacterial invasion of the tissues. When the progress of the disease is slow the ante-mortem multiplication of the invading bacteria may be remarkable. A case is described in which scattered throughout the surface and deep in the substance of the liver were spherical firm white areas about 5 mm. in diameter consisting of capsulated Gram-negative bacilli or bacteria suspended in a mucoid substance. Fresh material was not available for culture, but the organism had the morphological characters of *Bact. friedländeri*. The lung was extensively consolidated, and over large areas the alveoli were packed with capsulated Gram-negative bacilli.

Vaccination Against Influenza A. MELLANBY, H., ANDREWES, C. H., DUDGEON, J. A., and MACKAY, D. G. (1948). *Lancet*, 1, 978.

The vaccine used was a formolized influenza-A vaccine made from infected allantoic fluid and inactivated with 1 in 2,000 formalin. It was tested by measuring the antibody response to an intramuscular injection: 83% of adults showed more than a 4-fold rise in antibodies. The average rise was over 6-fold. The authors conclude that the vaccine did not produce any striking reduction in the incidence of influenza.

Comparative Efficiency of Rectal Swabs and Fecal Specimens in Detecting Typhoid and Salmonella Cases and Carriers. SHAUGHNESSY, H. J., FRIEWER, F., and SNYDER, A. (1948). *Amer. J. publ. Hlth*, 38, 670.

The results showed that when specimens containing many bacteria were examined neither of the methods was markedly superior to the other, but when the specimens contained apparently few organisms examination of the stool was more reliable than that of the swab. The post-cathartic specimens gave a greater percentage of positive results than the pre-cathartic. No single medium could pick up all intestinal pathogens.

BIOCHEMISTRY

Plasma Proteins in Pregnancy. MacArthur, J. L. (1948). *Amer. J. Obstet. Gynec.*, 55, 382.

The structure and function of the plasma proteins are outlined and the causes of hypoproteinaemia are enumerated. Hypoproteinaemia may be prevented by an adequate intake of biologically valuable protein. Until recently, protein was thought to be toxic to pregnant women and its intake was restricted. It is now, however, generally accepted that a high protein intake is desirable during pregnancy and will prevent the onset of toxaemia.

A simplified copper sulphate method of estimating plasma proteins was used in an examination of 600 normal pregnant women and a number with toxaemia. Comparison with haematocrit readings has shown that the fall in plasma protein level which takes place in normal pregnancy is due to haemodilution. Administration of hydrolysed protein has no effect on the plasma proteins in normal pregnancy. In cases of toxaemia of pregnancy, a marked difference is found between plasma protein level and the haematocrit reading. This amounted to 30% in cases of pre-eclampsia and was even higher in cases of eclampsia. Plasma protein levels rise rapidly after delivery in both normal and toxaemic patients. Protein hydrolysate given by mouth to toxaemic patients caused no rise in plasma protein level, and no improvement was noted as regards oedema or albuminuria. A similar observation was made when the hydrolysate was given intravenously, but reactions were common and the need to curtail fluid intake limited the amount of protein that could be given. Methionine was given to one patient with severe pre-eclampsia; the result was a rapid improvement in the condition and a rise in the plasma protein level. The author suggests that the hypoproteinaemia of toxaemia is due to liver damage. Methionine has a beneficial effect on the damaged liver in experimental animals, and it is suggested that it may prove of value in cases of toxaemia.

Josephine Barnes.

Reducing Substances in the Urine in Pregnancy and the Early Puerperium. Archer, H. E., and Haram, B. J. (1948). *Lancet*, 1, 558.

The reducing substance frequently present in the urine of pregnant women is often assumed to be lactose. This paper gives the results of tests for reducing substances on specimens of urine obtained throughout pregnancy and in the puerperium. Specific tests were made for the presence of lactose. Benedict's quantitative reagent was used, and if reduction occurred the ferric chloride test was made to exclude salicyluric acid. All specimens in which reduction occurred were then tested by a modified form of the methylamine reaction for lactose (Fearon, *Analyst*, 1942, 67, 30). Harwood stated that 0.1% lactose can be detected in the presence of 0.25% glucose, higher concentrations of glucose having a masking effect. The authors found that when the concentration of glucose was half that of lactose the methylamine reaction was less definite. When the amounts of lactose and glucose were equal negative results were obtained. The phenylhydrazine osazone test was carried out on all specimens with well marked reduction.

Of 123 antenatal patients, 118 (96%) had a reducing substance; of 777 specimens tested 501 (64%) contained reducing substance. Of 503 controls 54 (11%) had a reducing substance in the urine. Of 97 postnatal patients tested, 89 (92%) had a reducing substance in the urine, and of the 258 samples tested 210 (81%) contained the substance. The exact timing of the specimen in relation to meals was not possible. All specimens were passed in the morning. The amount of reducing substance in the antenatal group varied from a trace to 0.15%. Fourteen samples (1.8%) contained salicyluric acid. The rest all gave a negative result for lactose. Known diabetics were excluded from the control group. In the postnatal group 1.9% of the total number of specimens examined showed reduction due to salicyluric acid only. In 57% there was lactosuria, in many cases confirmed by osazone formation. Concentrations of ascorbic acid up to 100 mg. per 100 ml. urine did not reduce Benedict's reagent. Concentrations of 200 mg. per ml. reduced it slightly.

Lilian Raftery.

The Neutral Steroids of Human Urine: The Significance of the Various Steroid Fractions Excreted in Urine. Tompsett, S. L., and Oastler, E. G. (1948). *Glasg. med. J.*, 29, 133.

The total daily urinary excretion of the following steroids was estimated in a variety of cases: (1) 17-ketosteroids; (2) free reducing ketosteroids (corticosteroids); (3) pregnanediol; (4) total ketones of the neutral steroids; (5) non-ketones (alcohols) of the neutral steroids; (6) half-succinate esters of the non-ketonic fraction of the neutral steroids.

The cases investigated included 9 normal males, 7 males with various medical abnormalities, 3 normal females, 42 females with various medical abnormalities, and 6 surgical cases. The daily output of total neutral steroids in normal males ranged from 20.4 mg. to 41.2 mg. No significant variation was found in the male medical cases, with the exception of one case of malignant hypertension, in which the total neutral steroids rose from 16.3 mg. to 30.4 mg. during a severe attack. This rise was mainly confined to the non-ketonic fraction, which rose from 9.3 mg. to 21.3 mg. The daily output of total neutral steroids in the 3 normal females ranged from 15.6 mg. to 33.2 mg. Three cases of Simmonds's disease showed a very low excretion of total neutral steroids (3.8, 3.9, and 5.1 mg.). The 17-ketosteroids were very low. In one case of Cushing's syndrome there was a high total neutral steroid excretion (80 mg.), all fractions being increased. A case of adrenogenital syndrome had a total excretion of 110 mg., but the increase was confined mainly to the 17-ketosteroids. In all the surgical cases there was a rise in total neutral steroid excretion immediately after operation. The non-ketone fraction was mainly responsible for this. The authors also studied 5 women in the ninth month of pregnancy. The ratio of total ketones to 17-ketosteroids was much higher than in most other groups, except the surgical ones. The excretion of 17-ketosteroid was within normal limits, but the total neutral steroids were raised (range 53.6 to 193.2 mg.). It is suggested that the rise in total ketones is mainly due to 20-ketosteroids (pregnanolones) and that the catabolism of progesterone is through these.

R. Barer.

Excretion of Potassium in the Immediate Post-operative Period. Iob, V., Berry, R. E. L., and Campbell, K. N. (1948). *Univ. Hosp. Bull., Ann. Arbor*, 14, 57.

The renal excretion of potassium and nitrogen was studied in normal young men, in patients subjected to

herniorrhaphy, and in those subjected to abdomino-perineal resection of the rectum; all were receiving parenteral isotonic fluids, saline, glucose, or glucose-saline solution.

Potassium excretion was considerably increased in the immediate postoperative period, the increase being in proportion to the amount of trauma, but it was much more than could be expected from the breakdown of protoplasm. The most important factor was the necessary readjustment between intracellular and extracellular body fluids to the administration of intravenous liquids. Such adjustment is apparently accomplished at the expense of intracellular fluid, with its high potassium content. Contributory factors may be: (1) anaesthesia, (2) the sodium chloride concentration of the transfused fluid, (3) haemorrhage at operation, (4) dehydration, and (5) glycogenolysis.

The total potassium lost in these cases, although significant, is only a small fraction of the total cellular potassium. Signs of potassium deficiency were absent and there seemed no indication to reinforce parenteral solutions with potassium in short-term therapy.

T. J. Evans.

The Value of Combined Blood Phosphatase and Sedimentation Rate Determinations in the Diagnosis of Metastasis in Prostatic Carcinoma. BOYLAN, R. N., and TILLISCH, J. H. (1948). *J. Urol.*, 59, 931.

This is a study of 100 cases of carcinoma of the prostate gland with skeletal metastasis, in which the sedimentation rate (Westergren) and acid- and alkaline-phosphatase levels (King-Armstrong) had been determined. The authors also studied 65 cases of carcinoma of the prostate without metastases, diagnosed on clinical grounds. It was found that in the series with metastases the acid-phosphatase level was normal in 44% of cases, the sedimentation rate was normal in 23%, and the alkaline-phosphatase level was normal in 14%, but all 3 were normal in only 2% of cases. Of the 65 cases without metastases, the sedimentation rate was raised in 47.7%.

A. W. Badenoch.

HAEMATOLOGY

Urethane Treatment in Leukaemia. (Urethanbehandling ved leucose.) HANSEN, P. B. (1948). *Nord. Med.*, 38, 930.

Urethane was administered to 27 patients with leukaemia, in 21 of whom the leukaemia was of the chronic lymphatic type. In nearly every case the drug had to be withdrawn owing to its general toxic effects. The immediate results were good, and two-thirds of the patients improved. However, the authors consider that x-ray therapy is the treatment of choice; the toxic effects are less and the remissions longer.

Use of Folic Acid Derivatives in the Treatment of Human Leukemia. MEYER, L. M. (1948). *Trans. N.Y. Acad. Sci.*, 10, 99.

In 7 patients with chronic lymphocytic leukaemia and two with multiple myelomatosis the administration of teropterin (pteroyltriglutamic acid) produced a sense of well-being, but there was no objective improvement. In 3 of 5 patients with acute leukaemia short-lived remissions resulted from the administration of anti-folic-acid compounds.

The Nature of Anaemia in Leukaemia. COLLINS, D. H., and ROSE, W. McI. (1948). *J. Path. Bact.*, 60, 63.

The authors review 50 cases of leukaemia, with reference to severity and origin of the concomitant anaemias. Five patients are described in detail. Although blood loss and haemolysis may contribute to the anaemia, the authors believe that hypoplasia of the erythropoietic tissue in lymphatic leukaemia and defective formation in myelogenous leukaemia are the main causal factors.

Aleukemic Myelosis: Chronic Nonleukemic Myelosis, Agnogenic Myeloid Metaplasia, Osteosclerosis, Leukoerythroblastic Anemia, and Synonymous Designations. HELLER, E. L., LEWISOHN, M. G., and PALIN, W. E. (1947). *Amer. J. Path.*, 23, 327.

Three cases are reported. A study of this material and a review of the literature leads the authors to suggest that "aleukaemic myelosis" is in reality a variant of leukaemia, a view that is not generally held.

Enzymic Action of Viruses and Bacterial Products on Human Red Cells. CHU, C. M. (1948). *Nature, Lond.*, 161, 606.

The Detection of a Product of the Blood Group O Gene and the Relationship of the So-called O-substance to the Agglutinogens A and B. MORGAN, W. T. J., and WATKINS, W. M. (1948). *Brit. J. exp. Path.*, 29, 159.

Culture of Human Leukaemic Blood Cells *in vitro*; Technique and the Growth Curve. GUNZ, F. W. (1948). *Brit. J. Cancer*, 2, 29.

Culture of Human Leukaemic Blood Cells *in vitro*. Normal and Abnormal Cell Division and Maturation. GUNZ, F. W. (1948). *Brit. J. Cancer*, 2, 41.

The Skeletal Lesions in Leukemia. Clinical and Roentgenographic Observations in 103 Infants and Children, with a Review of the Literature. SILVERMAN, F. N. (1948). *Amer. J. Roentgenol.*, 59, 819.

Megaloblastic Anaemia of Pregnancy. Report of an Unusual Case. GILLESPIE, M., and RAMSAY, A. M. (1948). *Brit. med. J.*, 1, 828.

The authors describe a case which they consider is an example of megaloblastic anaemia of pregnancy. A few days after delivery profound anaemia was associated with leucopenia and a high proportion of immature granulocytes, which suggested the diagnosis of acute leukaemia. Sternal marrow puncture, however, revealed a myeloid: erythroblast ratio of 1.5: 1 and the presence of megaloblasts. Intensive treatment with liver extract and proteolysed liver was without effect and the patient died. At necropsy there was intensive hyperplasia of the marrow, the predominant cell being of "the primitive haemocytoblast variety" with many megaloblasts. The authors refer briefly to a somewhat similar case unassociated with pregnancy in which they believe that the precipitating factor was excessive dosage of a sulphonamide, 70 g. of which were given over a period of 5 weeks for acute tonsillitis. In neither case was folic acid administered.

Janet Vaughan.

Pernicious Anaemia of Pregnancy and the Puerperium. DAVIDSON, L. S. P., GIRDWOOD, R. H., and CLARK, J. R. (1948). *Brit. med. J.*, 1, 819.

The authors describe the successful treatment with folic acid of 3 patients with pernicious anaemia of pregnancy and one with Addisonian anaemia in relapse whilst pregnant. Three of these patients had previously failed to respond to potent liver extracts.

Anaemia Associated with Trauma and Sepsis. VAUGHAN, J. (1948). *Brit. med. J.*, 1, 35.

In this lecture the author considers the anaemias associated with sepsis, trauma (other than the direct effects of blood loss), and experimental sterile abscesses. Probably an interference with the synthesis of haemoglobin is the most important common factor. Amongst the evidence of disturbed metabolism is the low level of plasma iron and an increased excretion of nitrogen, but the exact way in which the metabolism of haemoglobin is affected is uncertain.

Studies on Free Erythrocyte Protoporphyrin, Plasma Iron and Plasma Copper in Normal and Anemic Subjects. CARTWRIGHT, G. E., HUGULEY, C. M., ASHENBRUCKER, H., FAY, J., and WINTROBE, M. M. (1948). *Blood*, 3, 501.

The authors studied 112 patients with various types of anaemia. In iron deficiency, infection, nephritis, and lead poisoning, where haemoglobin synthesis is disturbed, the level of free erythrocyte protoporphyrin was raised; in pernicious anaemia it was normal. The plasma iron was decreased in haemorrhage, rapid haemoglobin synthesis (as in pernicious anaemia during liver treatment), and in infection. It was increased in Cooley's anaemia, untreated pernicious anaemia, aplastic anaemia, and in the haemolytic anaemias. The plasma copper levels were generally constant.

Pernicious Anemia Caused by *Diphyllobothrium latum,* **in the Light of Recent Investigations.** VON BONSDORFF, B. (1948). *Blood*, 3, 91.

The author believes that the position of the worm in the small intestine is the most important factor in determining whether an infested subject develops a megalocytic anaemia. It is only when the worm is in the upper part of the small intestine that there is a risk of anaemia, due it is thought to its interfering with the interaction of the intrinsic and extrinsic factors.

Some Observations on Anaemia in Patients with Burns. BRAITHWAITE, F., and MOORE, F. T. (1948). *Brit. J. plast. Surg.*, 1, 81.

A New Hereditary Blood Disorder. Hereditary Methaemoglobinaemic Cyanosis. (Une nouvelle maladie héréditaire du sang; la cyanose méthémoglobinémique héréditaire.) CODOUNIS, A., LOUCATOS, G., and LOUTSIDES, E. (1948). *Sang,* 19, 65.

The authors describe the incidence of methaemoglobinaemia in 14 out of 103 relatives in 4 generations.

Osmometric Behaviour of Normal and Abnormal Human Erythrocytes. GUEST, G. M. (1948). *Blood*, 3, 541.

This paper should be read by all interested in the osmotic fragility test. The author demonstrates that in the normal, in congenital haemolytic jaundice, and in some patients with hypochromic anaemia the erythrocytes behave as almost perfect osmometers. In sickle-cell anaemia, in Cooley's anaemia, and in pernicious anaemia the cells were imperfect osmometers; this behaviour contributes to their increased resistance.

Iso-agglutinins in Cord Blood. JAKOBOWICZ, R., and BRYCE, L. M. (1948). *Med. J. Aust.*, 1, 669.

The authors examined the cord blood of 355 babies for anti-A and anti-B agglutinins. Generally the agglutinins in the foetus are very weak or absent. A case is cited in which the maternal antibody titre was 1 : 2,000, but no agglutinins were demonstrated in the cord serum. Possibly, however, antibodies of "immune" origin which the mother rarely develops are smaller in size and may cross the placenta more easily than the naturally occurring anti-A or anti-B.

Exogenous Hemochromatosis Resulting from Blood Transfusions. SCHWARTZ, S. O., and BLUMENTHAL, S. A. (1948). *Blood*, 3, 617.

The authors describe 5 patients of their own and review the clinical features and autopsy findings of a further 8 patients, all of whom had received large volumes of blood during life for various blood disorders. Marked haemosiderosis simulating haemochromatosis was observed. Cirrhosis of the liver occurs, and 3 of the authors' own patients had fibrosis of the pancreas; 2 patients developed diabetes.

The Significance of the Paucity of Sickle Cells in Newborn Negro Infants. WATSON, J. (1948). *Amer. J. med. Sci.*, 215, 419.

Only a small percentage of the erythrocytes of an affected infant will take on the sickle form, but by 4 months the proportion increases to 90%. The author suggests that the presence of foetal haemoglobin prevents the change to a sickle form.

The Life Span of the Sickle Cell and the Pathogenesis of Sickle Cell Anemia. SINGER, K., ROBIN, S., KING, J. C., and JEFFERSON, R. N. (1948). *J. Lab. clin. Med.*, 33, 975.

Erythrocytes with the sickle-cell trait survived normally in patients with sickle-cell disease, as judged by the Ashby technique. Erythrocytes from patients with sickle-cell anaemia were shown to have a shortened life span in subjects with the sickle-cell trait.

Studies in Hodgkin's Syndrome. VII. Nitrogen Mustard Therapy. ZANES, R. P., DOAN, C. A., and HOSTER, H. A. (1948). *J. Lab. clin. Med.*, 33, 1002.

Thirty-one patients were treated with nitrogen mustard and remissions were obtained in 21. All developed toxic reactions—initial anorexia, nausea and vomiting, and later leucopenia. Thrombocytopenia and skin eruptions were observed in about half of the patients.

Spontaneous Myohemoglobinuria in Man. Description of a Case with Recurrent Attacks. KREUTZER, F. L., STRAIT, L., and KERR, W. J. (1948). *Arch. intern. Med.*, 81, 249.

The literature concerning myohaemoglobinuria in man is reviewed. A new case of spontaneous myohaemoglobinuria, with muscular weakness and wasting similar to that in progressive muscular dystrophy, is described, the diagnosis being confirmed by spectrophotometric studies. Other studies of the blood and urine are reported.—[Authors' summary.]

Hemophilia: Current Theories and Successful Medical Management in Traumatic and Surgical Crises. WRIGHT, C.-S., DOAN, C. A., DODD, V. A., and THOMAS, J. D. (1948). *J. Lab. clin. Med.*, 33, 708.

The management of 43 patients observed over a period of 17 years is discussed. Blood transfusions, transfusions with freshly thawed frozen plasma, and the concentrated anti-haemophilic plasma fraction (fraction I of Cohn) have all been employed to reduce the coagulation time temporarily.

Treatment of Multiple Myeloma with "Stilbamidine." Clinical Results and Morphologic Changes. SNAPPER, I. (1948). *J. Amer. med: Ass.*, 137, 513.

In this paper Snapper records the results of stilbamidine treatment in 35 patients, twelve of whom had been treated for more than a year. In 80% of the patients the pain was relieved, but most of them relapsed within 12 months. The disease is no more than arrested temporarily, and the biochemical abnormalities are not affected. Four of the 12 patients treated for one year or more died; five were still ambulant.

A Coagulation Defect Produced by Nitrogen Mustard. SMITH, T. R., JACOBSON, O. C., SPURR, C. L., ALLEN, J. G., and BLOCK, M. H. (1948). *Science*, 107, 474.

The authors claim that the prolongation of the coagulation time produced by nitrogen mustard therapy is due to an excess of heparin, and may be reversed by the administration of toluidine blue or protamine.

The Heat-stabilized Sedimentation Rate. A Few Clinical Observations. (In English.) VANNFÄLT, K. (1948). *Acta med. scand.*, 129, 593.

Normally, citrated blood sediments considerably more slowly after 6 hours at 37° C. compared with a control sample kept in a refrigerator for the same length of time. This retardation is thought to be due to the formation at 37° C. of lysolecithin. In untreated pernicious anaemia the stabilization effect is much less marked than in treated cases. The results obtained in 132 patients, mostly with blood diseases, are recorded.

Hemolysis with Human Complement, Human Cells, and Tannic Acid: Application to Complement Fixation Test. THOMAS, L., and PECK, J. L. (1948). *Proc. Soc. exp. Biol.*, N.Y., 67, 475.

Tannic acid causes lysis of human erythrocytes in the presence of human complement. It is possible to use tannic acid in place of rabbit serum amboceptor as the indicator system in complement-fixation reactions.

N

An Experimental Study of the Comparative Efficacy of Heparin and Dicumarol in the Prevention of Arterial and Venous Thrombosis. KIESEWETTER, W. B., and SHUMACKER, H. B. (1948). *Surg. Gynec. Obstet.*, 86, 687.

Experiments are described concerning the effectiveness of heparin or dicoumarol in controlling thrombosis within artificially traumatized arteries and veins of dogs. Thrombosis occurs within 48 hours of injury, but anticoagulant therapy should be employed for longer than this, up to 2 to 3 weeks after repairs to arteries. Heparin and dicoumarol are both valuable; both should be used initially, and in the treatment of venous thrombosis heparin need no longer be administered when a satisfactory reduction in prothrombin concentration has been produced by the dicoumarol.

MORBID ANATOMY AND HISTOLOGY

Spread of Carcinoma to the Spleen. Its Relation to Generalized Carcinomatous Spread. HARMAN, J. W., and DACORSO, P. (1948). *Arch. Path.*, 45, 179.

Of 116 cases of carcinoma studied at necropsy, 30 were selected in which there was a strong tendency towards generalized invasion of the body, metastases having been found in organs in different body cavities. Cases in which tumour spread appeared to have been arrested by regional barriers or to have been halted in the pulmonary capillary bed were, however, excluded. At 9 necropsies metastases were visible in the spleen, and to these a further 6 cases were added in which microscopical metastases were discovered, giving a total incidence of 50%. Other organs in which nearly half the secondary deposits escaped naked-eye detection were: lungs, spleen, pancreas, heart, and bone marrow. Other figures of interest are given concerning metastasis to other organs. *W. S. Killpack.*

Aseptic Necrosis of Pancreas Due to Arterial Thrombosis in Malignant Hypertension. PAGEL, W., and WOOLF, A. L. (1948). *Brit. med. J.*, 1, 442.

The authors describe a fatal case of malignant hypertension with uraemia. At necropsy the pancreatic duct was normal, but the pancreas itself was swollen and hard owing to the presence of 10 small anaemic infarcts and 1 large one, each surrounded by a characteristic narrow haemorrhagic margin. There was no evidence of fat necrosis or of acute haemorrhagic pancreatitis. Histological examination revealed marked arteriosclerosis of the small arteries with partial or complete thrombotic occlusion of many of them.

The authors point out that neither the clinical nor the anatomical findings resembled in any way those seen in acute haemorrhagic pancreatitis; they doubt whether a vascular factor operates in the production of this disease. They rightly stress the existence of infarction of the pancreas as distinct from acute haemorrhagic pancreatitis. *R. B. T. Baldwin.*

Carcinoma in Chronic Gastric Ulcer: Results of Pathological Examination of 1,720 Resected Stomachs. (Über das Carcinom im chronischen Magengeschwür Ergebnisse der pathologisch-histologischen Untersuchungen an 1720 resezierten Mägen.) HOFFMANN. M. (1948). *Arch. klin. Chir.*, 260, 570.

While refuting the idea that simple gastric ulcer is a pre-cancerous condition, the author looks upon it as fertile ground for the development of malignant changes in a stomach with a carcinomatous diathesis.

Liver Biopsy in Sarcoidosis. SCADDING, J. G., and SHERLOCK, S. (1948). *Thorax*, **3**, 79.

Aspiration biopsies of the liver from 3 cases of sarcoidosis, and from a fourth case in which there was no other evidence of the disease, showed characteristic lesions.

Cytochemical Studies of Normal and Tumor Mast Cells in Tissues and *in Vitro.* PAFF, G. H., MONTAGNA, W., and BLOOM, F. (1947). *Cancer Res.*, 7, 798.

Histochemical studies of normal and tumour mast cells, and of tumour mast cells cultivated *in vitro*, reveal that the mast cells in all these categories contain lipids, cytochrome oxidase, and acid and alkaline phosphatases in their cytoplasm. The tumour cells also contain alkaline and acid phosphatases in their nuclei.—(Authors' summary.)

Melanin-forming Epidermal Tumours of the Skin: A Study of 57 Personally Observed Cases. STEWART, M. J., and BONSER, G. M. (1948). *J. Path. Bact.*, 60, 21.

The authors describe 57 pigmented epidermal tumours of the skin, including squamous papillomata, benign calcified epitheliomata, basal-cell carcinomata, and squamous carcinomata. The melanin in the tumours appears to be formed in the neoplastic epidermal cells. The authors conclude that the pigmented epidermal tumours are unrelated to the malignant melanoma or the benign pigmented naevus. Clinically these tumours behave like the corresponding non-melanin-forming epidermal tumours.

Pigmented Precancerous and Cancerous Changes in the Skin. KHANOLKAR, V. R. (1947). *Cancer Res.*, 7, 692.

An account is given of melanotic basal-cell and squamous-cell tumours of the skin. These are distinct from true melanomata and they are relatively benign. The author describes the abundance of dendritic pigment-forming cells (melanoblasts) in the growths. These cells are not easily seen in sections stained by the usual methods; they are more clearly shown by silver impregnation or by the "dopa" reaction. The author considers that the pigmented cells undergo hyperplastic proliferation in the tumours, "although they fail to keep pace with the increase in number of other epithelial cells and are later completely choked by them."

Benign Trophoblastic Cell Proliferation. TEDESCHI, C. G., and MATARESE, A. A. (1948). *Amer. J. Obstet. Gynec.*, 55, 758.

Two cases are described in which curettage was performed after early abortion. Microscopically, the tissue removed had many striking resemblances to chorionic carcinoma. On account of the finding of atypical cells, conservative treatment was adopted and both patients remained well. Diagnoses of syncytial endometritis and syncytioma were made in the two cases respectively. Detailed microscopical findings are described, and the authors suggest a method for deciding whether a mole is benign or malignant. *Braithwaite Rickford.*

Dendritic Cells. BILLINGHAM, R. E. (1948). *J. Anat., Lond.*, 82, 93.

During a reinvestigation of the anatomical basis of pigmentation of mammalian skin it was demonstrated that, although pigment granules are found in most ordinary epidermal cells of pigmented skin, they are not of endogenous origin but are derived from branched cellular elements called pigmented dendritic cells. These are located at the level of the basal layer cells of the epidermis. From them branches are given off which travel along the intercellular spaces between the ordinary epidermal cells, dichotomizing frequently, and ultimately terminating in the form of "caps" or "end-buttons" closely applied to the boundaries of ordinary epidermal cells. Dendritic cells have a cell-lineage of their own and are not derived from ordinary epidermal cells of the basal layer as a functional modification. Branched cellular elements which are similar in all respects to pigmented dendritic cells except that they lack melanogenic properties have been demonstrated in the non-pigmented epidermis of man, the guinea-pig, and the rabbit, and have been called white dendritic cells. It is suggested that dendritic cells fulfil some physiological function in the epidermis other than melanogenesis. It is concluded that the mammalian epidermis is a compound tissue composed of at least two distinct cellular elements, the dendritic cells and the ordinary epidermal cells.

From the Author's Summary.

Chronic Thyroiditis. MARSHALL, S. F., MEISSNER, W. A., and SMITH, D. C. (1948). *New Engl. J. Med.*, 238, 758.

A study of 187 cases of chronic thyroiditis has led to their separation into three groups. Group 1 comprised 41 (22%) specimens which showed a reaction seemingly due to infection: In 18, however, there were changes suggesting subacute inflammation—moderate fibrosis, numerous inflammatory cells, chiefly polymorphonuclear, and some degenerate acini with spillage of colloid causing a foreign-body giant-cell response. In a further 18 there was a more chronic stage of the process, in which the polymorphonuclears had largely or completely disappeared, but foreign-body giant cells were numerous, as also, scattered throughout the fibrotic stroma, were numerous lymphocytes and plasma cells. The remaining 5 cases illustrated the healed stage of the infective process with fibrosis, absence of giant cells, and paucity of other inflammatory cells. This last condition is commonly called Riedel's struma. In none of the stages in this infective process are there characteristic epithelial changes.

The second group was composed of 78 glands (42%) with changes typical of what is called Hashimoto's struma or struma lymphomatosa, there being marked infiltration of the stroma with lymphoid cells and atrophy and acidophilia of the epithelium. The glands were rubbery in consistence.

The third group of 68 cases (36%) undoubtedly included thyroiditis resulting from several causes. The basic microscopical changes were present but so mildly as to make further classification impossible. These cases were labelled "chronic thyroiditis, non-specific." Some in this group appeared to be of the "exhaustion atrophy" type, some may have represented early stages of the infective and lymphadenoid type, and others may have been due to vascular or traumatic causes.

Charles Donald.

Thyroiditis. CRILE, G. (1948). *Ann. Surg.*, 127, 640.

Subacute thyroiditis (27 personal cases) is a disease of unknown aetiology, but it may be due to a virus. Its onset is sudden, it may follow an upper respiratory infection, and it is six times as common in women as in men.

The striking feature of the histology is the presence of foreign-body giant cells, and adenomata are rarely present.

Struma lymphomatosa (14 personal cases) is also of unknown aetiology. It is neither the end-result of subacute thyroiditis nor does it progress to Riedel's struma. It appears to be part of a systemic disease. The gland does not become adherent to surrounding structures. It is firm, friable, avascular, and grey on section.

Riedel's struma (11 personal cases) is an inflammatory condition, often asymmetrical, with fibrosis which extends to involve surrounding organs. Its onset is insidious, and its progress slow and unaccompanied by systemic symptoms. Pressure on the trachea is the predominant symptom. The thyroid is stony hard, and difficult to distinguish from carcinoma. In the centre of many areas of Riedel's struma there is a degenerating adenoma.

During the period in which these cases were seen, 10 other cases of thyroiditis were encountered which did not fit into any category, and 3 cases of suppurative thyroiditis. Neither tuberculosis nor syphilis of the thyroid was observed.　　　　　　　　　　　　　　*R. S. Handley.*

Clinical and Pathological Features of Human Cerebral Necroses due to Irradiation. (Aspects cliniques et pathologiques des radionécroses cérébrales chez l'homme.) VAN BOGAERT, L., and HERMANNE, J. (1948). *Ann. Méd.*, 59, 14.

This paper reports 2 cases, the first in a child of 3 years and 3 months suffering from tinea and epilated by x rays, the second in a man of 61 with basal-cell carcinoma of the scalp treated by irradiation from 6 radium needles for 5 days.

Miliary Tuberculosis of the Aorta. (Die miliäre Aortentuberkulose. Betrachtungen anhand einer Miliärtuberkulose mit tuberkulöser Lebercirrhose.) WASER, P. (1948). *Schweiz. Z. Path. Bakt.*, 11, 29.

A case is reported in which a young adult showing radiologically a *primärkomplex* in his right lower lobe developed miliary tuberculosis and tuberculosis of his right sacro-iliac joint and died 4 months after the appearance of the first symptoms. Necropsy revealed tuberculous meningitis, tuberculous cirrhosis of the liver with nodules resembling Boeck's sarcoid, and miliary tuberculosis of the aortic intima. Histological examination of the aortic lesions showed nodules at the extreme inner surface of the intima projecting into the lumen; these arose just under the endothelium and pushed it up; small thrombi formed on its surface. Acid-fast bacilli were present in enormous numbers round the edges of the lesion.　　　　　　　　　　　　　　*C. L. Oakley.*

Fatty Liver Disease in Infants in the British West Indies. WATERLOW, J. C. (1948). *Spec. Rep. Ser. med. Res. Coun., Lond.*, 263, 5.

The clinical, pathological, and biochemical characteristics of fatty liver disease of infants in the British West Indies are described. This condition is differentiated from kwashiorkor, infantile pellagra, and a number of other conditions.

Fifteen cases of fatty liver disease were studied. Enlargement of the liver without oedema was found in 10% of an unselected group of infants. Liver biopsy showed the presence of fat, but the serum-protein concentration and dye clearance were normal. This condition was regarded as an early stage of fatty liver disease. Late cases in which the infant did not die or did not recover completely showed cirrhotic changes in the liver. Response to treatment was assessed by serial measurements of serum-protein concentration, the bromsulphalein test, and liver biopsy. In a small group of severely ill infants, methionine (5 g. daily for 7 days), choline (500 mg. daily for 4 to 7 days), and inositol (1 g. daily for 4 to 7 days) had no beneficial effect. High milk intake resulted in improvement.

　　　　　　　　　　　　　　A. C. Frazer.

Infantile Cortical Hyperostoses. (In English.) VAN ZEBEN, D. (1948). *Acta paediatr., Stockh.*, 35, 10.

In three cases of infantile cortical hyperostoses there were the characteristics found by Caffey—cortical thickenings in the bones and tender swellings deep in the soft tissues, though the peculiar facial expression caused by the latter in the region of the lower jaw, as described by Smyth, was not evident. In addition, a familial incidence was encountered (2 of the patients were siblings and the third was a cousin) and an anterior convex curvature of the thickened tibiae was seen radiologically. In each case the presenting symptom was swelling of the legs, associated in 2 cases with pain and impaired movement. Biopsy in one case revealed infiltration with osteoblasts, lymphocytes, and marrow cells. Culture and inoculation tests were negative. Two of the infants recovered; the third died of an intercurrent infection. *W. F. Gaisford.*

Investigations on the Pathology of the Pancreas in the First Year of Life, with Special Reference to the Connective Tissue. (Untersuchungen zur Pathologie des Pankreas in ersten Lebensjahr unter besonderer Berucksichtigung des Bindegewebes.) ULE, G. (1948). *Frankfurt. Z. Path.*, 59, 359.

In 72 premature infants and infants within the first year of life the weight and length of the pancreas differed greatly. The pancreas at this age is very rich in connective tissue and there is a relative insufficiency of the pancreas. Signs of embryonal haematopoiesis were frequently seen. In cases of general infection hyperaemia was repeatedly found, and in 3 cases haemorrhages. Cases are described in which severe alterations (phlegmonous pancreatitis, obstruction of duct) had occurred without corresponding clinical signs *in vivo.*

Histologic Studies on a Virilizing Tumor of the Adrenal Cortex. WEBER, E. J., and MENTEN, M. L. (1948). *Amer. J. Path.*, 24, 293.

The patient, a boy of $3\frac{1}{4}$ years, showed precocious development of the genitalia and the hair of the face, axillae, and pubic area, and his daily excretion of 17-ketosteroids was 14.7 mg., thus nearly approaching the adult level. A large right-sided adrenal tumour was removed and the output of ketosteroids fell to that normal for a child of $3\frac{1}{4}$—about 5 mg. daily. The cells of the tumour contained fatty secretory globules stainable with ponceau fuchsin or Sudan IV, and a series of cells were identified with a range of secretory changes comparable with those seen in the normal adrenal cortex.

　　　　　　　　　　　　　　R. A. Willis.

Hydatidiform Mole in the Fallopian Tube. CHALMERS, J. A. (1948). *J. Obstet. Gynaec. Brit. Emp.*, **55**, 322.

A review of the literature on hydatidiform mole in the Fallopian tube is given and a case is described, bringing the total number recorded to 15. There was a history of 10 weeks' amenorrhoea followed by irregular bleeding and lower abdominal pain. A diagnosis of ectopic pregnancy was made. At operation a small swelling 1 cm. × 2.5 cm. was found in the middle of the right Fallopian tube. There was a small rupture in the tube and a small amount of free blood in the pouch of Douglas. The pelvic organs were otherwise normal. The Aschheim-Zondek reaction carried out after the operation was negative. Microscopical examination revealed avascular oedematous villi and areas of trophoblastic proliferation.

Gladys Dodds.

Comparison of Histological Characters of Autumnal Maritime Encephalitis and Japanese B Encephalitis. (In Russian.) GRASHCHENKOV, N. I., GLAZUNOV, I. C., and ROBINSON, I. A. (1948). *Nevropat. Psikhiat.*, **17**, No. 1, 5.

Clinical and epidemiological observations were made in over 200 cases of encephalitis; description of the histological features is based on 39 necropsies. The histopathological features in the first days of autumnal encephalitis consisted, apart from the typical exudative and proliferative phenomena, of marked degenerative changes and of numerous foci of softening in all sections of the brain. The inflammation is often of haemorrhagic type, variable in extent in different cases; haemorrhages in the cerebral meninges were particularly extensive. The proliferative phase is characterized by the proliferation of the elements of the endothelium of blood vessels; this is accompanied by the formation of infiltrates, mainly in the adventitial spaces, and by proliferation of histiocytes and mononuclears. With few unimportant exceptions, a similar picture was seen in cases of Japanese B, encephalitis. Although the latter disease occurs mainly in summer or towards its end, the authors concluded, on the basis of histological evidence, that autumnal maritime encephalitis and Japanese B encephalitis are identical. It had been proved earlier by Russian workers that the two viruses are identical.

H. P. Fox.

Leishman's Stain Adapted for Use with Histological Sections. BLACK, R. H. (1948). *Ann. trop. Med. Parasit.*, **42**, 52.

Thin paraffin sections, brought down to distilled water, were treated as follows: saturated aqueous solution of picric acid, 5 to 10 minutes; wash with water; Leishman stain (1 : 2 water) 25 minutes; wash in running water, 10 to 15 minutes; dehydrate through xylol-acetone mixtures—xylol 5%, 30%, 70%—to pure xylol; mount in neutral medium.

Erythrocytic malarial parasites are well stained and exoerythrocytic forms of *P. gallinaceum* in sections of liver, kidney, and spleen are clearly shown, but there is no differential staining of the chromatin and cytoplasm of the parasites. The tissue cells, especially of cellular organs (liver, kidney, intestine, spleen) are well stained.

J. F. Corson.

Rapid Cytodiagnosis in Dermatology. (Le cytodiagnostic immédiat en dermatologie.) TZANCK, A. (1948). *Ann. Derm. Syph.*, Paris, **8**, 205.

The examination of stained scrapings from skin lesions can in certain cases assist in diagnosis. Material for examination should be obtained by scraping the base of the lesion and not from crusts or exudations. The stain normally used has been the May-Grünwald-Giemsa. Accuracy in interpretation of the findings can be obtained only by study of many specimens. The method is of value in the diagnosis of all types of cutaneous cancer.

SERUM PROTEINS: A Review

BY

J. R. MARRACK AND H. HOCH

From the Department of Chemical Pathology, London Hospital Medical College

CONTENTS

For many years it has been realized that serum globulin is not a single homogeneous protein, but comprises a variety of proteins which have different physical, chemical, and physiological properties. The salt fractionation methods that have been used for many years do not yield fractions which are sharply distinguished one from another in any properties. A striking example is that of the fractionation of diphtheria antitoxic serum with ammonium sulphate; antitoxin is found in a series of fractions ranging from that precipitated between 30 and 35% saturation to that precipitated between 52 and 56% saturation (Barr and Glennie, 1931).

During recent years new methods have been used for the separation and study of the proteins of serum and plasma. In particular the moving boundary method of electrophoretic analysis developed by Tiselius (1937) raised great hopes, for by this method at least five fractions of different mobility can be recognized in human serum: the mobilities of these fractions are distinct and little material of intermediate mobility can be

detected. Cohn and his colleagues at Harvard have studied the factors that affect the solubility of proteins. On the basis of these studies they have developed a method of separation of proteins by independent variation of these factors. This method has revealed the presence in serum of a great variety of distinct proteins. It has now been used extensively and on a large scale for the preparation of active fractions relatively free from other protein. Methods have been devised by which the molecular weights of protein molecules can be calculated and tentative estimates can be made of their shapes and sizes.

The separation of fractions by electrophoresis is based on one property only, that is, mobility in an electric field. The molecules contained in a fraction which, on the basis of mobility, is homogeneous, may have different shapes, weights, and sizes, different chemical compositions and different physiological properties. The globulin fractions separated by Cohn's methods and, still more, those separated by salt fractionation, contain proteins of different mobilities. The grouping of

serum globulins, therefore, depends on the choice of property on the basis of which they are classified.

The mobilities of the components of the fractions prepared by Cohn's methods have been measured. A very large number of abnormal sera have been studied by electrophoresis; from these results it is possible to draw conclusions as to the changes of electrophoretic pattern that may be expected in various types of disease, and the significance of these changes. Few abnormal sera have been studied by the other new methods. We will, therefore, use the electrophoretic patterns as the basis of discussion in this article.

Comprehensive reviews of investigations into the concentration, distribution, and significance of proteins in serum have been published by Janeway (1943), Stern and Reiner (1946), Luetscher (1947), and Gutman (1948).

I. Electrophoresis: Method and Interpretation

The object of electrophoresis is to demonstrate the presence of constituents which have different mobilities and to measure the relative concentrations of these constituents. The conditions of pH and salt concentration influence the results and they have to be specified. The range of pH commonly used for plasma or serum is between 7.7 and 8.6; in this pH range all serum proteins carry negative charges and therefore move towards the anode. The salt concentration usually chosen is equivalent to an ionic strength of 0.1 or 0.2.* The experiments are made in the special apparatus designed by Tiselius which consists of a U-tube with a rectangular cross section, the upper ends of which are connected to two electrodes. The solution to be analysed is run into the bottom part of the U-tube, buffer solution is placed on it and in the remaining space, including the electrode vessels.

At the beginning there is one boundary between the protein solution and the supernatant buffer in each limb of the U-tube (Fig. 1a). On passing an electric current these two boundaries move away from the original positions at velocities equal to the velocities of the protein ions below these moving boundaries. If several protein constituents of different mobilities are present the original boundaries will split into several boundaries moving with different speeds. In addition to these moving boundaries there are two almost stationary boundaries, δ and ϵ, which are due to changes in conductivity at the positions of the original boundaries (Fig. 1). Before it is put in the U-tube the protein solution is dialysed against the buffer. In order to reduce to a minimum convection currents arising from differences in temperature within the U-tube between the inner and the marginal parts

* The ionic strength is defined by $\frac{1}{2}(c_1z_1{}^2+c_2z_2{}^2+ \ldots)$ where $c_1, c_2, \ldots$ are the ion concentrations and $z_1, z_2, \ldots$ are the valencies.
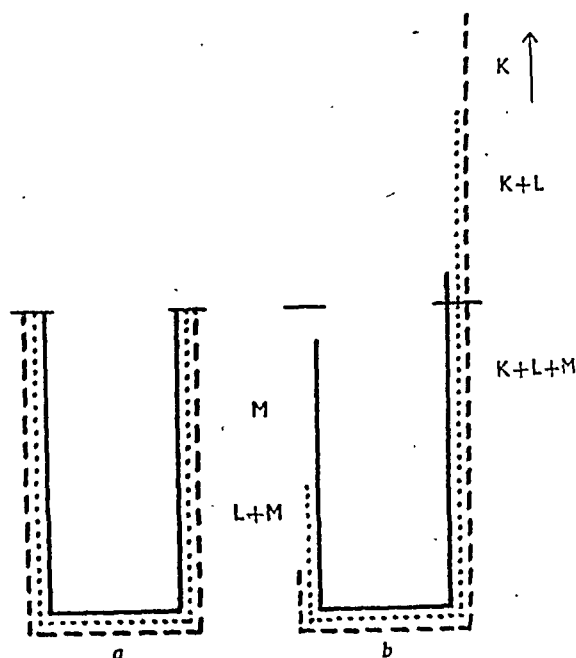
FIG. 1.—Distribution of three components, K, L, and M, of different mobilities: (a) Before electrophoresis; (b) after electrophoresis.

during the passage of the current, the U-tube is immersed in a water-bath of a temperature near the density maximum of the solution (0–4° C.).

The methods of observing the number and positions of the boundaries and of measuring the relative concentrations of the fractions are based on the change of refractive index produced by the change in the concentration of protein at the boundary. On account of this change of refractive index a horizontal ray of light passing through the tube is deflected downwards. The extent of the deflection depends on the rate of change of the refractive index. In the space immediately above and below the boundary the concentration does not change and a ray of light passing through the tube is not deflected. In Thovert's cylindrical lens (Thovert, 1914; Philpot, 1938; Svensson, 1939) and in Longsworth's *Schlieren* scanning (1939) methods the vertical deflection is translated by optical and mechanical devices into a lateral deflection which is recorded on a screen. The boundary appears on the screen as a peak. On migration the sharpness of the peak changes. Variation in conductivity and in pH have each the effect of sharpening the peak in one limb and of broadening it in the other. These effects may be in the opposite or in the same direction. Spreading by diffusion is superimposed in both limbs. If a boundary peak remains single during migration over a long distance it is usually inferred that the protein is electrophoretically homogeneous. But this need not necessarily be the case, since a mixture of similar proteins, the mobilities of which are distributed in a smooth frequency curve, can also show a single peak after

migration over a long distance. Criteria to define the degree of non-homogeneity have been proposed. (For literature see Alberty, 1948.)

Fig. 2 shows the concentration distribution along the vertical direction in a boundary. The difference between the concentrations at A and that at A′ is proportional to the difference between the refractive indices at A and that at A′. This can be shown to be proportional to the area under the peak ADA′. When the protein solution used is a solution of serum pro-
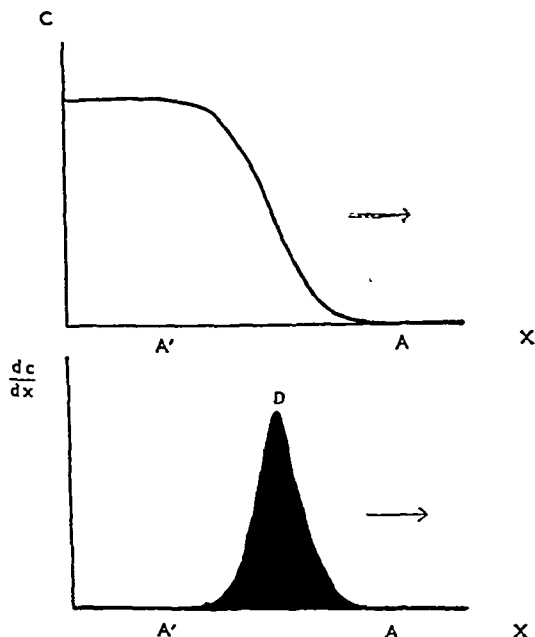
FIG. 2.—Concentration-distribution in a boundary, and corresponding peak formed on plate.

teins the components that have different mobilities are represented by peaks as in Fig. 3. They are called albumin, a_1- (Longsworth, 1942), a_2-, β- and γ-globulin. In order to separate the a_1-globulin from the albumin a diethybarbiturate buffer of pH 8.6 is used (Longsworth, 1942). When the phosphate buffer is used at a pH lower than 8.0 the a_1-globulin peak does not separate from the albumin peak; the "albumin" therefore includes a_1-globulin.* Subsequently it has been shown that each component is composed of a group of proteins the mobilities of which differ slightly. Frequently two peaks can be distinguished in the region of the β-globulin boundary. The faster has been called β_1- and the slower β_2-. A small component normally seen in human sera between the β_2- and the γ-globulins has been designated as β_3-, but is now called γ_1- (Deutsch *et al.*, 1946). If plasma is used the fibrinogen is represented is a peak, $\varnothing$, which includes

the γ_1-globulin and lies between the β- and γ-globulin. The concentrations of these components are proportional to the areas (measured with a planimeter) under these peaks; the relative concentrations are calculated from the relative proportions of these areas, and, if the total concentration of protein in serum has been determined, the absolute concentrations of these fractions can be calculated.* This simple statement needs some qualification. In the first place it assumes that the rate of change of refractive index with change of concentration (specific refractive increment) is the same for each protein and that no gradients of refractive index are produced across the boundaries by concentration gradients of other substances. In the second place, the molecules which move in groups and cause the changes of refractive index are not simple proteins, in the sense of compounds built of amino-acids, but are aggregates of protein, carbohydrate, and lipids more or less firmly united. The assumption that the specific refractive increment is the same for each protein is approximately correct if the concentration of "protein" used as a basis of calculation is the weight of the total protein-lipid-carbohydrate aggregate that moves as a unit. But the traditional methods of reckoning amounts of proteins are based on the amounts of nitrogen that they contain, or on the dry weight of the protein-carbohydrate complex left after extraction of the lipids. As the amounts of lipid in the a- and β-globulins of serum are larger than the amounts in the albumin and γ-globulin, the relative proportions of a- and β-globulin calculated from the areas of the peaks are higher than would be found if the proportions were calculated from the amounts of nitrogen in these fractions. This difference in method of reckoning accounts for part of the difference between the albumin/globulin ratios found by salt fractionation and by electrophoresis; the difference becomes large when the amount of lipid in the serum is high. Other factors affect the apparent concentrations and mobilities. All that need be said here is that for comparable results ionic strength and protein concentrations must be kept within certain limits. Experiments to determine these limits have been made by Perlmann and Kaufman (1945), Svensson (1946), Koenig *et al.* (1946), and others.

II. Fractionation by Precipitation Methods

It is not generally practicable to use electrophoresis as a routine method of examination. When interest was focused on the reduction of serum proteins in nephrosis it was sufficient to measure the total concentration of proteins. Now that it is realized that in some diseases the concentration of albumin may be very low, although the total concentration of protein is not reduced,

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*Throughout this review we will use the term " a-globulin" (without a numerical suffix) to mean a_1-globulin separated at a pH not exceeding 8.0; in these instances, the a_1-globulin is included in the "albumin."

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*The reproducibili-y of the measurements can be assessed from the data given by Dole (1944), Seibert *et al.* (1947), and others. The error in the estimate of the albumin is usually within $\pm 2\%$; that of any other component, if 10 or 20% of the total, within $\pm 5\%$, depending on the resolution of the peaks.

and that the concentration of one electrophoretic fraction of the globulin may be changed without changes of the concentrations of the others, the relation of fractions separated by suitable routine methods to electrophoretic fractions must be considered.

Salting Out.—The fractions separated by precipitation with strong salt solutions are not homogeneous. In the earliest investigations Tiselius (1937) found that the globulin fraction, precipitated from horse

pattern very little. (In the serum studied little protein, Howe's "euglobulin,"* was precipitated by this concentration of sulphate.) · When the concentration was raised to 17.4% about one-half of the γ-globulin, a quarter of the β-, and none of the α-globulin was precipitated. This fraction includes Howe's pseudoglobulin I. All the γ-, three-quarters of the β-, and one-quarter of the α-globulin including Howe's pseudoglobulin II were precipitated by 21.5% sulphate. The residual solution, containing Howe's "albumin," contained α- and β-globulin. Dole (1944)

TABLE I

DISTRIBUTION OF ELECTROPHORETIC COMPONENTS (g./100 ml. OF ORIGINAL SERUM) IN FRACTIONS PRECIPITATED WITH AMMONIUM SULPHATE FROM NORMAL HUMAN SERUM (NEURATH ET AL., 1947)

		Albumin	Globulin			
			α_1	α_2	β	γ
Whole Serum		3.52	0.4	1.07	0.80	1.41
Fractions Precipitated Between						
Molar Concentrations	Saturation (%)					
0–1.4	0–34	0	0	0.04	0.09	0.62
1.4–1.7	34–42	0.05	0	0.22	0.5	
1.7–2.1	42–51	0.10	0.12	0.33	0.08	0.09
2.1–	51–	2.28	0.38	0.22	0.20	0

serum by 30% saturation with ammonium sulphate, contained some albumin, and that 25% of the protein not precipitated by 50% saturation was globulin. Cohn, McMeekin, and colleagues (1940), and Svensson (1941) found that the fractions of the globulin precipitated between various concentrations of ammonium sulphate contained all three of the electrophoretic components. The distribution in the fractions precipitated from human serum are given in Table I. Jager and Nickerson (1948) found that about three-quarters of the globulin precipitated by 33% saturated ammonium sulphate was γ-globulin; the precipitate also contained 11 to 14% of β-globulin and 5 to 16% of α_1-, and α_2-globulin. The heterogeneity of these salt-precipitated fractions is also shown by the fact that a β-globulin, which contains the mid-piece of complement, makes up a considerable part of the globulin precipitated from human and guinea-pig serum by 34% saturated ammonium sulphate (1.39M.) at a pH of about 6.1 (Pillemer et al., 1941, 1943).

In Kekwick's (1940) method of preparing γ-globulin from normal human serum the precipitate formed with 12% sodium sulphate contained 5% of β- and α-globulin. In the course of their study of the fractionation of normal human sera by Howe's (1921) method Gutman and his colleagues (1941) found that 13.5% sodium sulphate changed the electrophoretic

also found that Howe's albumin contained more than one globulin fraction.

In other studies of human serum, such as that of Kibrick and Blonstein (1948), the amounts of protein · precipitated by various concentrations of sodium sulphate have been compared with the amounts of the fractions found by electrophoresis. The fact that the amount of protein precipitated by a given concentration of a salt is equal to the amount of some electrophoretic fraction does not imply that this precipitate is electrophoretically homogeneous.

Low Salt Concentration.—The amount of globulin precipitated from serum by dilution or dialysis against solutions of low salt concentration depends on the pH and ionic strength. Thus the so-called "end-piece" of guinea-pig complement is not precipitated at pH 5.2 and an ionic strength of 0.075 but is precipitated at this pH when the ionic strength is reduced to 0.01 (Pillemer et al., 1941). Erickson et al. (1947) found that in euglobulin, precipitated from syphilitic serum by dilution and the passage of CO_2 or by addition of HCl to pH 6.0–6.1, about one-half was γ-globulin, about a quarter α-, and a quarter β-globulin.

*The term "euglobulin" has been used promiscuously to mean the fraction that is insoluble at low salt concentrations, the fraction precipitated by ⅓ saturation with ammonium sulphate, and that precipitated by 13.5% sodium sulphate. If the term is used the precise meaning should be specified.

TABLE II
PARTITION OF SERUM PROTEINS (g./100 ml.) BY HOWE'S METHOD (GUTMAN *ET AL.*, 1941)

	Total Protein	Albumin	Globulin			
			Eu–	Pseudo–I	Pseudo–II	Total
Mean	7.2	5.2	0.2	1.3	0.5	2.0
Standard Deviation	0.35	0.25	0.11	0.23	0.16	0.27
Range	6.5 to 7.9	4.7 to 5.7	{ under 0.1 to 0.4 }	0.8 to 1.9	0.2 to 0.8	1.3 to 2.4

Quantitative Methods.—The ratio of albumin to globulin found by salt fractionation, by Howe's method, for example, is higher than that calculated from the electrophoretic patterns. Thus Dole and Braun (1944) found that the ratios measured electrophoretically were roughly two-thirds of the ratios found by Howe's method. This is due to two causes. First, the amount of globulin that is not precipitated by the critical concentration of salt (21.5% sodium sulphate in Howe's method) is more than the amount of albumin that is precipitated at this concentration. Second, the estimates of protein in the salt fractions are actual estimates of the amounts of nitrogen in these fractions: the estimates of the electrophoretic fractions, calculated from the areas of the peaks, include lipids which contain little nitrogen. As the globulins contain more lipid than the albumin the ratio of globulin to albumin calculated from the areas under the peaks is higher than the ratio based on nitrogen contents. When the electrophoresis is run at pH about 7.8 the α_2-globulin is included in the albumin, and the ratio of albumin to globulin then agrees more closely with that found by the usual salt-fractionation methods.

Attempts have been made to devise simple precipitation methods by which the relative concentrations of the fractions separated will agree better with those estimated by electrophoresis. Milne (1947) and Kibrick and Blonstein (1948) recommend the use of 15.75, 19.90, and 27.20% solutions of sodium sulphate for precipitation. Twenty volumes of these solutions are added to one volume of serum, and the final concentrations are then 15, 19, and 26%. The amount of protein precipitated by 15% was called γ-globulin :

the difference between the amounts precipitated by 19 and 15%, β-globulin ; the difference between the amounts precipitated by 26 and 19%, α-globulin ; and the protein left in solution, albumin.

The amount of γ-globulin estimated in this way in 14 sera agreed well with the amount of electrophoretic γ-globulin. In only two sera was the difference greater than 0.2 g./100 ml. Out of 10 sera the estimates of β-globulin by the two methods differed by less than 0.2 g./100 ml. in all but one serum, and the estimates of α-globulin by less than 0.3 g./100 ml. in all but one. The authors do not mention whether the electrophoresis was run at pH 7.8 or 8.6, but the amounts of electrophoretic α-globulin given are those that would be expected if it included both α_1- and α_2-globulin.

Pillemer and Hutchinson (1945) introduced a method of precipitation with methyl alcohol in the cold. They found that the amount of protein precipitated agreed well with the amount of globulin estimated by electrophoresis. However, Nitshe and Cohen (1947), using this method, found in 23 normal sera a ratio of albumin to globulin (2.25), which is much higher than any found by electrophoresis. Martin and Morris (1949) have confirmed that the albumin/globulin ratio found by precipitation with 26% sodium sulphate and by the methyl alcohol method agree with that found by electrophoresis at pH 8.0, whereas the ratios found by precipitation with sodium sulphite (Campbell and Hanna, 1937) and magnesium sulphate (Popjak and McCarthy, 1946) did not.

Low-Temperature-Low-Salt-Low-Dielectric-Constant Fractionation.—The separation of proteins in this

TABLE III
DISTRIBUTION OF ELECTROPHORETIC FRACTIONS (g./100 ml. OF ORIGINAL PLASMA) SEPARATED BY THE LOW-TEMPERATURE-LOW-SALT-LOW-DIELECTRIC-CONSTANT-FRACTIONATION METHOD

Fraction	Ethanol Concentration (%)	pH	Ionic Strength	Temperature (°C)	Globulin				Fibrinogen
					Albumin	α	β	γ	
I	8–10	7.2	0.14	−3	0.2	0.3	0.5	0.3	2.1
II and III	25	6.8	0.09	−5	0.8	1.1	9.1	7.0	1.9
IV ± 1	18	5.2	0.09	−5	0	4.5	0.5	0.1	0
IV 4	40	5.8	0.09	−5	0.9	2.7	2.2	0	0
V	40	4.8	0.11	−5	29.9	1.3	0.3	0	0

Descending

Ascending

3A.—Electrophoretic pattern of normal pooled sera diluted to 1.4 g./100 ml. Diethyl barbiturate buffer, pH. 8.6, ionic strength 0.1. 80 min. at 9 v/cm. Concentrations (g./100 ml.): total protein, 7.1; albumin, 3.70 (52) * ; α_1–globulin, 0.55 (8); α_2–globulin, 0.80 (11); β–globulin 1.00 (14); γ–globulin, 1.05 (15).
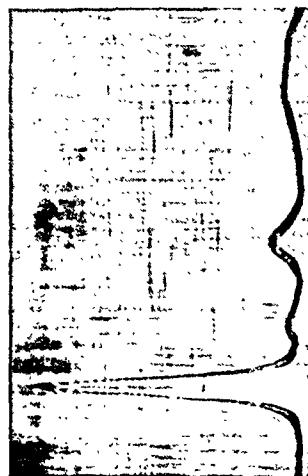
3B.—Electrophoretic pattern of normal serum (blood donor) diluted to 1.2 g./100 ml. Phosphate buffer, pH. 8.0., ionic strength 0.1. 74 min. at 9.3 v./cm. Concentrations (g./100 ml.): total protein, 7.3; albumin+α–globulin, 4.5 (61); α_2–globulin, 0.55 (7); β–globulin, 1.00 (14); γ–globulin, 1.30 (18). In diethyl barbiturate buffer, pH. 8.6, ionic strength 0.1, this serum, at same dilution, gave albumin, 3.90 (53); α_1–globulin, 0.40 (5.5); α_2–globulin, 0.65 (9); β–globulin, 1.00 (14); γ–globulin, 1.35 (18.5).

FIG. 3.—Electrophoretic patterns of normal pooled sera and normal serum (blood donor).

* Figures in brackets denote percentag: of total protein.

process, developed by Cohn and his colleagues, depends on the fact that small variations of the ionic strength or dielectric constant of solutions may have a great effect on the solubility of protein molecules: this effect depends on the electric configuration which may be highly characteristic of certain molecules. The dielectric constant is lowered by the addition of ethyl alcohol. The five variables—namely, ionic strength, concentration of protein, pH, temperature, and dielectric constant—can be varied independently. The main fractions obtained are given in Table III. Fractions II+III and IV−4 are further broken down into sub-fractions. A variety of proteins that appear homogeneous have been isolated and proteins that have physiological activity have been allocated to the various fractions (Tables VI and VIII). As appears from Table III, this method does not separate proteins according to their mobilities, but various refined proteins have thus been prepared for therapeusis. These include albumin, which contains little salt; globulin, containing antibodies to measles, whooping-cough, diphtheria, scarlet fever, etc.; fibrinogen for making fibrin foam, a haemostatic; and fibrin film, an absorbable membrane. A full description of the method and the theory on which it is based is given by Edsall (1947).

III. Normal Concentrations of Serum Proteins

Total Protein.—Estimates of the normal concentrations of proteins in serum differ widely. The means reported by some authors are below the lower limits reported by others. This may be accounted for, in part, by a difference of activity and of posture of the subjects. Thus Perera and Berliner (1943) found that the total protein in the serum of normal ambulant subjects was about 0.8 g./100 ml. more than after they had been in bed for some hours. During recent years more attention has been paid to the technique for estimating nitrogen, and most estimates of concentrations in the serum of healthy persons who are up and about approximate to those of Gutman et al. (1941) given in Table II. The effect of posture should be born in mind when the concentrations in the serum of bedridden patients are considered.

The proteins of serum are considered in this discussion, but proteins are often estimated in plasma. If oxalate is used as an anticoagulant, water diffuses out of the erythrocytes to compensate for the increased concentration of salt in the plasma. This water dilutes the plasma slightly, and consequently the concentration of protein found in oxalated plasma is usually no higher than that found in serum, although the plasma contains about 0.3 g./100 ml. of fibrinogen whereas the serum contains none.

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It should be noted that these concentrations are calculated by multiplying the protein-nitrogen by the conversion factor 6.25.* To get the concentration of protein-lipid-carbohydrate complex the average factor for use with normal serum should be 6.73 (Armstrong et al., 1947), but the use of this factor is undesirable as the amount of lipid varies.

Electrophoretic Fractions.—Examples of the proportions and amounts of the fractions separated by electrophoresis are given in Table IV. In the estimates made at pH 7.8 the albumin includes α_1-globulin. Dole's subjects were healthy young male adults, but Armstrong's samples were drawn from pools of plasma from blood donors. The concentrations of the globulin fractions reported by Dole and by Lewis and McCullagh are lower than those reported by Seibert and colleagues. The mean concentration of γ-globulin according to Dole differs from the mean of Seibert et al. by almost two standard deviations. This difference must be borne in mind when the concentrations of the globulin fractions in abnormal sera are considered.

Serum Proteins in Pregnancy and Infancy.—After about the twenty-second week of pregnancy the serum protein falls by an average amount of 0.8 g./100 ml. (Plass and Matthew, 1926; Oberst and Plass, 1932, 1936; Hoch and Marrack, 1948). The albumin alone is reduced, and this reduction runs parallel to the reduction of the concentration of haemoglobin in the blood occurring at the same time; both are attributed to increase of the blood volume. After delivery the albumin rises.

The serum proteins of babies at birth are low and variable; mean concentrations range from 5.11 (Rapoport et al., 1943) to 5.52 (Darrow and Cary, 1933). Lower values are found in the serum of premature babies. The concentration rises slowly during the first six months (Hickmans et al., 1943), and adult levels are reached by the third year (Trevorrow et al., 1941–2).

Longsworth et al. (1945) reported electrophoretic analyses of the plasma or serum of foetal blood drawn from the umbilical cord and of maternal blood taken one hour after delivery. In the maternal plasma or serum the average concentration of albumin was low (3.56 g./100 ml.) and the concentration of β-globulin raised (1.62 g./100 ml.). In foetal plasma or serum the albumin (3.82 g./

*Various methods, such as the biuret and tyrosine methods are standardized by nitrogen estimations.

TABLE IV

RELATIVE PROPORTIONS AND ABSOLUTE CONCENTRATIONS (g./100 ml.) OF ELECTROPHORETIC FRACTIONS IN
NORMAL PLASMA AND SERUM

	pH	Percentage of Total Protein						Concentration (g./100 ml.)						Total Protein (g./100 ml.)	A/G
		Alb.	α_1	α_2	α	φ	γ	Alb.	α_1	α_2	β	φ	γ		
Armstrong *et al.*, (1947). Mean of 20 pools of plasma...	8.6	55.2	5.3	8.7	13.4	6.5	11.0								
Dole (1944). Plasma of 15 young male adults. Mean	8.6	60.3	4.6	7.2	12.1	5.1	11.0	4.04	0.31	0.48	0.81	0.34	0.74	6.7*	1.53
S.D.		2.8	0.7	1.3	1.9	0.6	2.5	0.27	0.051	0.083	0.126	0.059	0.151		0.181
Seibert *et al.* (1947). Serum of 43 normal adults. Mean	8.5	53.3	8.0	10.4	13.8		14.2	3.88	0.58	0.76	0.01		1.05	7.29†	1.15
S.D.		2.6	1.35	1.38	1.89		2.68	0.23	0.10	0.10	0.14		0.22	0.35	0.12
Range		47.6–	5.3–	7.7–	7.9–		8.7–	3.4–	0.38–	0.57–	0.73–		0.59–	6.3–	0.91–
		59.2	11.5	12.9	11.6		19.7	4.3	0.87	0.94	1.38		1.46	8.2	1.45
Moore *et al.* (1941). Plasma of 25 normal adults Mean	7.8	62.5		7.5	13.1	5.0	11.9								
Lewis and McCullagh (1944). Plasma of 21 normal adults. Mean	7.8	62.7		7.2	13.1	5.4	11.7	4.09		0.47	0.81		0.77	6.51*	
Range		60.1–		6.0–	11.0–	2.8–	8.6–	3.72–		0.39–	0.65–		0.55–	5.94–	
		67.2		8.7	15.9	7.2	14.8	5.11		0.66	1.07		0.91	7.82	

* Estimation by Kjeldahl. † Estimation by Biuret method.

100 ml.) and γ-globulin (0.97 g./100 ml.) were higher and other globulin fractions lower than in the maternal samples.

The sera of new-born calves, lambs, and foals contain very little γ-globulin. The colostrum contains a globulin which resembles the γ-globulin of serum (Smith, 1946). After the new-born animals have ingested colostrum, γ-globulin appears in their serum, but ingestion of colostrum after the calves have reached 24 hours of age does not lead to an increase of γ-globulin (Hansen and Phillips, 1947). It seems that during the first day of life this globulin is absorbed without being split up by digestive enzymes.

Pedersen (1944) found that about 20% of the total protein and 50 to 80% of the globulin in the serum of newborn calves is a new protein which he called "fetuin." The molecular weight of this protein is 51,000, which is less than that of albumin. The serum of foetal foals and lambs also contains fetuin.

IV. Composition, Physical, and Physiological Properties

Composition.—This is discussed under the headings amino acids, lipids, and carbohydrate.

Amino Acids.—Complete estimates of the

amino acids of the albumin, α-, β-, and γ-globulin, and fibrinogen of human serum have been compiled by Edsall (1947) from the work of Brand, Kassel, and Saidel (1944), Brand (1946), and Shemin (1945). The chief points of interest are the very low content of tryptophane and the relatively low content of glycine in albumin. The contents of various groups of amino acids run in the following orders:

Basic: Albumin $>$ α-globulin $>$ β-globulin $>$ γ-globulin.

Di-carboxylic: α-globulin$>$albumin$>$$\beta$-globulin $>$$\gamma$-globulin.

Hydroxy: Albumin$<$$\alpha$-globulin$<$$\beta$-globulin $<$ γ-globulin.

Lipids.—Blix, Tiselius, and Svensson (1941) found that the α- and β-globulin of normal human serum contain considerable quantities of cholesterol and phospholipids (Table V). Reckoning the phospholipid as lecithin the total amounts (averages of three normal sera) in these fractions were 11.7 and 18.7%. Edsall (1947) considers that the amounts of cholesterol found in albumin and γ-globulin indicate that these fractions were impure, as Cohn, Strong *et al.* (1946) found under 0.04% and 0.06% of cholesterol in purified albumin and γ-globulin.

TABLE V

LIPID AND CARBOHYDRATES, AS MANNOSE (g. per 100 g.), IN ELECTROPHORETIC FRACTIONS OF HUMAN SERUM[*]

Electrophoretic Fractions	Normal			Pneumonia		
	Cholesterol	Lipid-Phosphorus	Carbohydrate	Cholesterol	Lipid-Phosphorus	Carbohydrate
Albumin	1.07	0.09	1.15	0.71	0.02	5.8
α–Globulin	4.45	0.29	6.0	0.42	0.21	9.9
β–Globulin	8.67	0.40	6.2		0.56	1.5
γ–Globulin	0.41	0.04	3.0	0.28	0.12	3.7

[*] Adapted from Blix, Tiselius, and Svensson (1941).

By the special methods developed by the Harvard school two lipoproteins have been isolated from human serum (Table VI). One, with the mobility of α-globulin, contains 10% of nitrogen, 16% of cholesterol, and 35% of total lipid ; the other, with the mobility of β-globulin contains only 4% of nitrogen, 35% of cholesterol, and 75% of total lipid. The conversion factors, by which the nitrogen in these two lipoproteins should be multiplied to obtain the weight of lipoprotein, are 10 and 25.

Carbohydrate.—Table V shows that there is more carbohydrate in the α- and β-globulin of human serum than in the albumin and γ-globulin. There is some confusion about the amounts of carbohydrate in albumin and other components that are precipitated by the higher concentrations of ammonium and sodium sulphate. Hewitt (1937) prepared a fraction from horse serum which he called "globoglycoid." Rimington and Van den Ende (1940) consider that this is a complex of albumin and globulin. They prepared two albumins from ox serum, both of which contained very little carbohydrate ; one crystallized in hexagonal plates and the other in needles. McMeekin's (1940) horse serum albumin that contained 5.5% of carbohydrate crystallized in hexagonal discs ; his carbohydrate-free albumin crystallized in rods ; the two albumins had the same molecular weights.

Besides true albumin the filtrates obtained after precipitation of globulin contain two proteins, seroglycoid and seromucoid, which contain large amounts of carbohydrate.. Hewitt's (1937) seroglycoid from human serum contains 8.9% and seromucoid 25% of carbohydrate. Both are not coagulated by heat, but seroglycoid is carried down with the coagulum when it is heated with coagulable proteins. Hewitt (1938) believes that the amount of seroglycoid in human serum is of the order of 0.4 g./100 ml. These carbohydrate-rich proteins have received little attention in the

most recent investigations. An $α_2$-globulin, which contains 4.3% of carbohydrate and has the solubility of albumin, has been isolated (Surgenor, Strong *et al.*, 1948).

Molecular Weights.—When a solution of a protein is spun in an ultracentrifuge, a boundary zone, above which the solution is free from this protein, travels down the column of solution. If solutions of proteins which have different sedimentation rates are spun, the boundaries can be observed as peaks, as in electrophoresis ; the area under a peak is proportional to the concentration of the corresponding protein. Sedimentation constants, calculated from the distance that the boundary sinks in a given time, are reckoned in Svedberg units (S). The sedimentation constants of molecules depend on their weights, shapes, and densities. Other things being equal the sedimentation rate rises with the molecular weight: a spherical molecule has a higher sedimentation rate than a flat or elongated molecule. The molecular weight of a protein can be calculated from the sedimentation rate, diffusion constant, and partial specific volume ; also from the distribution of the molecules, when they have attained equilibrium in a gravitational field, and from the osmotic pressure. If it is assumed that the molecules have a certain shape the ratio of the long to the short axis can be calculated. In Table VI it is assumed that the molecules, other than the β-lipoprotein, are prolate spheroids (that is, cigar-shaped) and that the β-lipoprotein is approximately spherical.

The ultracentrifuge has been used mainly to measure the sedimentation constants of isolated proteins. A difficulty arises, however, in the interpretation of the sedimentation patterns found with whole human serum, but not with the serum of other animals. Four peaks are found (Pedersen, 1945). One small peak corresponds to a heavy fraction with a sedimentation constant. between

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TABLE VI

PHYSICAL PROPERTIES OF FRACTIONS ISOLATED FROM HUMAN SERUM (ONCLEY, SCATCHARD, AND BROWN, 1947)

Fraction	Sedimentation Constant	Partial Specific Volume	Molecular Weight	Calculated Dimensions, Å
Albumin ...	4.6	0.733	69,000	150 × 38
α_1–globulin, lipoprotein	5.0	0.841	200,000	300 × 50
α_2–globulin	9	0.693	(300,000)	
β_1–globulin	5.5	0.725	90,000	190 × 37
,,	7	0.74	(150,000)	
,,	20	0.74	(500,000 to 1,000,000)	
lipoprotein ... ·	2.9	0.950	1,300,000	185 × 185
β_2–globulin ...	7	0.739	(150,000)	
γ–globulin ...	7.2		156,000	235 × 44
,,	10		(300,000)	

17 S and 20 S. A larger peak corresponds to the bulk of the globulin with a sedimentation constant of about 7 S. A third peak corresponds to the albumin, and a fourth, that of the " x-component,' merges with the albumin peak. As the concentration of the serum rises the proportion of the protein in the form of the x-component rises. The reason for the relatively slow rate of sedimentation of this component is not the relative smallness of its molecules, but their low density, which is due to a high concentration of lipid (Pedersen, 1945). Pedersen considers that the x-protein is a complex formed by albumin, globulin, and lipid. It disappears when the lecithin of serum is broken down by the lecithinase of *Cl. Welchii* (Peterman, 1946). The lipoproteins of Table VI account for only about 5% of the total protein of serum, whereas the x-protein may amount to 50% or more of the total protein in undiluted sera. Blix and Pedersen (1947) think that the x-protein " must in the main be composed of other fractions than the β-lipoprotein." The question then arises as to whether the fractions, isolated even by such gentle methods as electrophoresis, are actually separate in serum. The globulin fractions with the sedimentation constants 5.0, 5.5, 9, and 10 S cannot be detected in the sedimentation patterns of whole serum.

Immunology

Antibodies.—Studies of the mobilities and molecular weights of antibodies are reviewed by Kabat (1943). In the rabbit sera that have been studied antibodies have been found in the γ-globulin and have molecular weights of the same order (about 160,000) as that of the bulk of the γ-globulin. In horse sera, antibodies may have the mobility of γ-globulin, or mobilities ranging from that of the faster γ-globulin (γ_1-globulin)

to that of the slower β-globulin (β_2-globulin); the T-fraction (Van der Scheer, Wyckoff and Clarke, 1940) has a mobility between those of β- and γ-globulin. The molecular weights of the horse-serum antibodies to protein antigens are of the same order as that of the bulk of the γ-globulin. Antibodies to polysaccharides (such as those of pneumococci) may have much higher molecular weights of the order of 900,000 or molecular weights intermediate between this and that of normal γ-globulin.

The fractions II—1, 2, and 3 of human serum separated by Cohn's methods, of which 98% is γ-globulin by electrophoretic standards, contain a large number of antibodies effective in diphtheria, influenza, mumps, whooping cough, scarlet fever, poliomyelitis, lymphocytic choriomeningitis, and against certain streptococci and vaccinia. Typhoid H-agglutinin is found mainly in fractions II—1, 2, and 3 ; typhoid O-agglutinin and the isohaemagglutinins in fraction III—1 (Edsall, 1947). According to Deutsch et al. (1946) the isohaemagglutinins and typhoid O-agglutinin are found mainly in their γ_1-globulin. The anti-syphilitic antibody also has a mobility between those of β- and γ-globulin according to Davies et al. (1945); Erickson et al. (1947) also placed most of the activity in the faster γ-globulin, but found some activity in the slower β-globulin.

In the sera of human beings and monkeys antibodies to pneumococcal polysaccharides have molecular weights of about 160,000. However, haemagglutinins in rabbit sera (Paič, 1939) and the isohaemagglutinins of human sera (Pedersen, 1946) have molecular weights of the order of 1,000,000. The anti-syphilitic antibody, extracted from the floccules formed with Kahn antigen, had two components with sedimentation constants corresponding to molecular weights of

about 1,000,000 and 160,000 ; there was more of the lighter component, but the heavier was more active serologically (Davis *et al.*, 1945).

As already noted the concentration of γ-globulin in the serum of a new-born baby is rather higher than that in the mother's serum. The baby's serum also contains any antibodies present in the mother's serum in about the same concentration. On the other hand no antibodies can be detected in the sera, which contain little γ-globulin, of newborn calves, lambs, and foals.

Generally it may be said that antibodies in human serum are found in the γ-globulin or in a fraction with a mobility between those of β- and γ-globulin, and that the antibodies to all protein antigens, as far as is known, have molecular weights of the same order as that of the greater part of normal serum globulin. Boyd and Bernard (1937) suggested that all γ-globulin may be antibody of some sort. Kabat (1943) points out that the amount of antibody in the serum of non-immune persons is small. The largest amount, reported in a human being who had recently recovered from pneumonia, was about 1 mg. of antibody protein/ml. (Kabat, 1939) ; no antibody could be detected in the serum of the same person several months later. Absorption of antibodies with homologous streptococci from the serum of patients with rheumatic fever, when the antistreptolysin O titre was raised, did not change the relative concentrations of the γ-globulin or other serum fractions significantly (Dole, Watson, and Rothbard, 1945). When animals are immunized the serum globulin increases, but by no means all this increase is active antibody (Marrack, 1938). It is probable that a large proportion of the 8 mg. or so of γ-globulin in 1 ml. of human serum is such inert globulin, which may be called "reaction" globulin.

Serum Proteins as Antigens.—Rimington and van den Ende (1940), using the guinea-pig uterus technique, found no antigenic difference between the two forms of albumin that they prepared from ox serum and that the albumin, seroglycoid and seromucoid reacted as distinct antigens. The albumin of human serum does not cross-react with other serum proteins. Chow (1947) has used antiserum for estimating albumin in serum and found concentrations which agreed with those obtained by electrophoresis at *p*H 8.4. The immunology of the seroglycoid and seromucoid of human serum has not been studied.

The question arises whether the various components of any one of the three fractions, α-, β-, and γ-globulin of human serum, are antigenically similar, although they differ in other properties,

and whether proteins, similar antigenically, may occur in different electrophoretic fractions.

The immunological method recently introduced by Oudin (1946) shows that horse serum contains at least eight distinct antigens ; it is not possible to recognize which are the antigens that are detected. It is possible that two of these are seroglycoid and seromucoid. This would leave albumin and five globulin antigens. In horse serum antigenic differences were found between the high-molecular weight antibodies to bacterial polysaccharides, diphtheria antitoxin, and a γ-globulin of high molecular weight prepared from normal horse serum, all of which had the mobility of γ-globulin : β-globulin formed some precipitate with an antiserum to high-molecular-weight antibody (Treffers *et al.*, 1941, 1942). On the other hand, in rabbit serum, which resembles human serum more than horse serum does, no antigenic differences were detected between normal γ-globulin, antibody to egg-albumin, and antibodies to bacterial polysaccharides (Treffers and Heidelberger, 1941).

Kendall (1937) prepared a fraction of human globulin by precipitation methods, and called it "α-globulin," but identified with the γ-globulin of Tiselius. We have confirmed by electrophoresis in the Tiselius apparatus (Marrack, Johns, and Hoch, 1949) that this "α-globulin" is mainly γ-globulin ; preparations usually contain less than 6% of faster fractions. Kendall prepared antiserum to his "α-globulin." After absorbing this antiserum with other globulin fractions he used it for estimating the amount of this fraction in serum by the quantitative precipitin method. The concentrations he found were about double the amounts of γ-globulin found by electrophoresis. Jager *et al.* (1948) made antisera by immunizing with γ-globulin prepared by Cohn's method and found similar high concentrations. However, they did not absorb their antisera with other globulin fractions. On the other hand, Kibrick and Blonstein (1948) immunized rabbits with γ-globulin, free from other electrophoretic components ; the antisera were absorbed with albumin and with β- and α-globulin. The estimates of γ-globulin in human serum made with these antisera agreed with the estimates made by their method of fractionation with sodium sulphate, and these, again, agreed with the estimates by electrophoresis.

By Kendall's method a small fraction only of the total γ-globulin of serum is recovered as Kendall's "α-globulin." We have found (Marrack, Johns, and Hoch, 1949) that γ-globulin, separated

by electrophoresis, and Kendall's " α-globulin " gave identical precipitation curves with some antisera to Kendall's " α-globulin." Estimates, with these antisera, of the γ-globulin in normal human serum agreed with estimates made from the electrophoresis pattern. We, therefore, detected no antigenic difference between different fractions of the γ-globulin. However, estimates of γ-globulin in normal serum made with other antisera to Kendall's " α-globulin " were considerably higher than those found by electrophoresis. The type of reaction suggested a cross-reaction between antibodies to γ-globulin and other globulin fractions rather than the presence in the antisera of antibodies to antigens other than γ-globulin. In view of Kendall's results and the cross-reaction which Treffers *et al.* (1942) found between horse β- and γ-globulins we are doubtful whether β- and γ-globulins can be regarded as antigenically completely distinct. On the other hand, the C-reactive protein, mentioned later, has the mobility of α-globulin but is a distinct antigen. Members of the same electrophoretic fraction may therefore be antigenically different.

Complement.—Four components are recognized in complement. C′1 (mid-piece) is heat labile and

al., 1941), and of the C′1, C′2, and C′4 of human serum (Pillemer *et al.*, 1943, Ecker *et al.*, 1945). It appears that C′2 and C′4 form a complex in guinea-pig serum, but not in human serum. If the electrophoretic fractions of guinea-pig serum are analogous to those of human serum, the C′1 is a β-globulin and the C′2-C′4 complex an α-globulin. The C′1 of human serum is a β-globulin, most of which has a sedimentation constant close to that of the greater part of the serum globulin. About half the protein in the fraction that contained C′2, isolated from human serum, was albumin ; the slower component in the fraction had a mobility close to that of β-globulin.

The two fractions, C′1 and C′2-C′4, isolated from guinea-pig serum together made up about 0.5 mg./ml. of serum ; this is slightly more than the amount of protein that Heidelberger (1941) found was added to an immense precipitate when it absorbed complement from 1 ml. of guinea-pig serum. A high proportion of the protein in these preparations from guinea-pig serum may, therefore, be active complement. The amount of complement in human serum seems to be of the same order. Properties of the components of human and guinea-pig complement are given in Table VII.

TABLE VII

PROPERTIES OF COMPONENTS OF COMPLEMENT

Subject		Resistance to Heat	Mobility (*p*H 7.7)	S	Polysaccharide (g./100 ml.)
Guinea-pig	C′1 ...	Heat labile	2.9×10^{-5}	6.4 (major component)	2.7
	C′2 ... C′4 ...	Heat labile Heat stable	} 4.2×10^{-5}		10.3
Human	C′1 ...	Heat labile	2.9×10^{-5}	6.9 (major component)	
	C′2 ...	Heat labile	2.6×10^{-5}		3

insoluble in dilute salt solution (ionic strength below 0.1) at a *p*H of about 5.2. C′2 (end-piece), also heat labile, is distinguished from C′1 as it does not become insoluble until the ionic strength is reduced to 0.01. C′3 is relatively heat stable and can be removed from serum by adsorption on a polysaccharide prepared from yeast and by other means. C′4 is heat stable and is destroyed by ammonia and other amines that can combine with aldehyde groups. By fractional precipitation with ammonium sulphate and at a low *p*H and ionic strength, fractions have been isolated that contain a considerable proportion of the C′1 and of the C′2-C′4 complex of guinea-pig serum (Pillemer *et*

Isolated γ-globulin is anti-complementary. The sera of a number of diseases (particularly malaria, leprosy, and kala azar) give false positive reactions with the Wassermann and other tests for syphilis. In most of these sera the concentration of γ-globulin is abnormally high (Cooper *et al.*, 1946). Volkin *et al.* (1947) used the euglobulin, separated by dilution and acidification, for these tests. They claim that crude albumin fractions from normal sera inhibit false positives and do not inhibit true reactions. More recently they (Volkin *et al.*, 1949) have ascribed this inhibitory action to a phospholipid contained in the albumin fraction. Kabat (1946) regards with suspicion such methods of dis-

tinguishing true from false reactions. He thinks that the procedures inhibit weak and do not inhibit strong reactions, so that the effect is merely to distinguish between strong and weak reactions. We may compare the degree of inhibition by haptenes of precipitate formation with homologous and heterologous antigens.

Physiological Properties

The distribution of physiologically active or important substances in the fractions separated by the low-temperature-low-ionic-strength-low-dielectric-constant method is given in Table VIII.

TABLE VIII

CONSTITUENTS AND PROPERTIES OF FRACTIONS SEPARATED BY LOW-TEMPERATURE-LOW-SALT-LOW-DIELECTRIC-CONSTANT FRACTIONATION METHOD

Fraction	Constituent Fractions	Properties
I	Fibrinogen Anti-haemophilic globulin	

II + III	γ-globulin
	β_1-globulin, including lipo-protein
	
	β_2-globulin
		Numerous antibodies
		Carries cholesterol
		   phospholipids
		   vitamin A
		   carotenoids
		   steroid hormones
		complement, midpiece
		Prothrombin
		Typhoid O-agglutinin
		Iso-haemagglutinins

IV—1
 	
IV—4
	α_1-globulin, including lipo-protein
	β_1-globulin, lipid free
	
	α_2-globulin
		Carries cholesterol
		   phospholipids
		   some steroids
		Thyreotropic hormone
		Idoprotein
		Metal-combining protein
		Carries bilirubin
		Serum esterase
		Hypertensinogen

V	Albumin α_1-globulin	Carries bilirubin ,, ,,

The components with the lowest and highest mobilities serve familiar purposes. The γ-globulin contains antibodies. About 80% of the colloid osmotic pressure of serum is due to albumin, as it is present in the largest amount and has the lowest molecular weight. It is therefore essential for the maintenance of the plasma volume. Albumin has other special properties: it adsorbs a variety of substances which are little or not at all adsorbed by the globulin fractions other than α_1- and a carbohydrate-rich fraction of the α_2-globulin. The

most familiar of these is bilirubin. Some bilirubin is also carried by the α_1- and α_2-globulin (Martin, 1949), and Cohn (1948) states that a protein in the α-globulin fraction is the only one that carries the bilirubin that gives an indirect Van den Bergh reaction. A large number of other substances are adsorbed almost exclusively by serum albumin. For example, about 20% of phenol-sulphone-phthalein in serum is free ; the rest is adsorbed to albumin. The dye Evan's blue (T-1824) used for estimating plasma volume is carried by the albumin. This is the basis of the method of estimating Evan's blue devised by Morris (1944). Sulphanilamide and the sulphone drugs are adsorbed also. This adsorption must be taken into account in all comparisons of the concentration of a substance in the plasma with its rate of excretion in the urine. When the concentration of the albumin in the plasma is lower than normal, it may be expected that an abnormally high proportion of an adsorbable substance will be free.

A protein which has the special property of combining with metals has been crystallized (Koechlin, 1949). It has the mobility of β_1-globulin, and contains 1.8% of carbohydrate. Its molecular weight is 90,000 (S=5.0). The complex formed with iron has a salmon red colour (Schade, 1946) with maximum absorption at 465 mμ. The amount of complex formed is measured by the light absorption at this wave length. At a pH above 6.5 1 g. of this protein will combine with 1.25 mg. of iron ; this is two atoms of iron per molecule of protein (Surgenor, Koechlin, and Strong, 1949). The protein also combines with copper and with zinc. The maximum amount of copper, 2 atoms per molecule, is bound near pH 8.5. Below pH 8 iron will displace copper from the complex. Surgenor *et al.* estimate that 100 ml. of normal serum contains 0.24 g. of this protein, capable of binding 0.3 mg. of iron. This estimate agrees with the maximum binding capacity (0.315 mg./100 ml.) found by Rath and Finch (1949).

The anti-haemophilic globulin can be precipitated from normal serum by dilution and acidification. The plasma of haemophilics clots in normal time when a solution of this globulin is added to it.

The α- and β-lipoproteins are particularly interesting. These proteins may serve merely as vehicles for lipids which are insoluble in water or they may have other functions.

V. Serum Proteins in Disease

Most of the changes of serum proteins, revealed by any method, except the immunological, are not specific. In most investigations the cases studied

have not been followed through the course of the disease and the changes found in the serum proteins have not been correlated with the clinical condition or with other chemical changes. Surveys of the investigations that have been made are apt to degenerate into a series of variations of ways of saying " M.N. (194x) found the relative concentration γ-globulin raised in P. disease."

Unfortunately many authors report the relative concentrations of electrophoretic components without mentioning the total concentration of protein. The albumin concentration falls in most conditions in which protein concentrations are abnormal ; a rise in relative concentration of the globulin components may merely reflect this reduction of albumin. The changes that occur in disease can be grouped under the following headings : Response to infection or injury ; effects of deficiency of protein ; changes due to excess of lipid in the serum ; changes associated with disease of the liver ; changes in the serum of patients with myelomatosis. Examples of electrophoretic patterns in disease are given in Fig. 4 (pp. 188–191).

Response to Injury and Infection.—The changes in the concentrations of albumin and globulin in serum provoked by bacterial toxins are illustrated

acute infections are reduction of the albumin, with a rise in the α-globulin during the acute stage and a rise in the γ-globulin continued during the later stages and convalescence.

The most thorough studies of the electrophoretic changes in acute infections are those of Dole and his colleagues, in which they made repeated examinations of the serum. They used barbiturate buffer at pH 8.6 and therefore separated α_1-globulin from albumin. Unfortunately in two of their reports they gave relative concentrations only, displayed on charts. It is however possible to deduce the changes of absolute concentration when the changes of relative concentration are more extreme. In three cases of scarlet fever (Dole, Watson and Rothbard, 1945) in which arthritis did not occur, the ratio of albumin to total protein fell and remained low for six or more weeks after the temperature had fallen to normal. The relative and absolute concentrations of α_1-globulin were raised : in two of the cases the concentrations remained high for seven weeks after the onset. There was an absolute increase of the α_2-globulin in two cases, highest in the first two weeks, and a moderate relative increase in a third. In two cases the relative concentration of γ-globulin was moderately raised. In the third a purulent infec-

TABLE IX

CHANGES OF PLASMA PROTEINS AFTER INJECTION OF T.A.B. VACCINE. (LOHR AND LOHR, 1922)

Before Injection	Temperature	Proteins (g./100 ml.)			
		Total	Albumin	Globulin	Fibrinogen
After first injection 3½ hours 2 days	100°	7.34 7.48	4.94 4.53	1.99 2.48	0.41 0.47
After second injection 2 days	102°	7.04 8.59	4.35 4.18	2.10 3.68	0.69 0.73
Before third injection After ,, 3¼ hours 2 days	102°	8.57 ,, 8.56	3.58 ,, 3.75	3.96 ,, 4.08	0.73 ,, 0.73

by Löhr's (1921) study of the effects of injection of T.A.B. vaccine (Table IX). The concentration of albumin fell and that of globulin rose. The changes did not reach their height during the first reaction and progressed as the injections were continued. Similar changes that have been found in various infections will be mentioned later.

Electrophoretic Patterns in Acute Infections.— As far as any generalization is possible from the limited number of cases reported, it may be said that the changes in the electrophoretic patterns in

tion of the right maxillary antrum and ethmoid sinuses was found at the end of the second week. At this time the temperature rose, and the relative concentration of γ-globulin rose to about three times the normal level and the antistreptolysin O-titre to about 1,000. The infection of the sinuses cleared up under treatment in two days. The γ-globulin and titre then sank but were still high about five weeks later.

The concentrations after 3½ hours are the same as before injection.

In three cases (J.K., G.S., and F.H.) in which rheumatic fever followed scarlet fever the relative concentrations of α_1-globulin were raised in the early stages. In one case the absolute concentration was raised. Both relative and absolute concentrations of α_2-globulin and γ-globulin were raised and in two cases the relative concentration remained high throughout the 160 days during which the sera were studied. In two of the cases (J.K. and G.S.) a detectable increase of the relative concentration of α_2-globulin followed the cessation of sulphadiazine therapy and preceded a rise of temperature and the onset of pains in the joints in the fourth and fifth weeks. In the serum of G.S. the relative α_1-globulin also rose. In the serum of J.K., who had a purulent nasal discharge from the seventh to the eighteenth day, the relative γ-globulin rose to about three times the normal and at the same time the antistreptolysin O-titre rose to over 2,000. The titre then fell, though less rapidly than the relative γ-globulin concentration. In the other two cases the titre was low. It is an interesting point that patient J.K. had a large boil on the leg in the eleventh week. The white blood count then rose and the temperature and erythrocyte sedimentation rate rose slightly, but no change of the serum proteins was detected except a doubtful increase of the relative concentration of α_1-globulin. In these cases the relative concentration of β-globulin was low or little changed.

In a case of typhus fever (Dole *et al.*, 1947) the first sample of blood was taken on the fifth day of illness before the rash appeared and before the Weil-Felix reaction was positive. The main changes were those of the albumin and γ-globulin. The total protein was low in this sample, remained low for about three weeks and then rose above normal to about 8.5 g./100 ml. The absolute concentration of albumin was low throughout the seven weeks during which the serum was examined. The α_1-globulin rose slightly at the end of the second week. The α_2- was high from the start and stayed high throughout. The relative γ-globulin was over twice the normal average in the first sample of serum, rose to well over three times the normal and was still three times the normal at the end of seven weeks. Since the total protein rose the absolute concentration must have risen to about four times the normal average. In the cases of scarlet fever and rheumatic fever there was some evidence of a relation between the presence of specific antibodies and the rise of γ-globulin. In this case the γ-globulin was over

double the normal average before specific antibodies were detected.

In most other studies by electrophoresis the *p*H has been under 8.0. The α_1-globulin has, therefore, not been separated from the albumin. In their early studies Longsworth *et al.* (1939) called attention to the increase in α-globulin in the early stages of disease. Blix (1939) found that the absolute concentration of α-globulin was raised in six out of seven cases of pneumonia and remained high after lysis. The highest figures were about three times the normal level given by Blix. The γ-globulin was not appreciably raised except in one case, and this patient died. Luetscher (1941) also found the α-globulin raised to about twice the normal in one of two cases of pneumonia.

Rutstein and others (1945) found that the absolute and relative concentrations of γ-globulin were increased in cases of rheumatic fever both during attacks and during quiescent periods. The α-globulin was increased less constantly. In two cases, examined during an acute attack, both α- and γ-globulin were about double the normal. In 12 cases of rheumatic fever Malmros and Blix (1946) found that the α-globulin was over 0.8 g./100 ml. in all cases at the first time of examination. The β-globulin was normal. The γ-globulin was increased in all, the maximum being 3.4 g./100 ml. The same authors found that the α-globulin was raised up to 0.7 to 1.0 g./100 ml. in cases of tonsillitis, and the increase persisted after the attack subsided. The absolute concentration of γ-globulin was appreciably raised in one of four cases during the attack and in all but one case after the attack.

In malaria transmitted by mosquitoes the liver is invaded by the malarial parasites at the beginning of the infection (Shortt *et al.*, 1948), and there is considerable evidence of liver damage in glandular fever. The changes found in the acute stages of these diseases may be, in part, due to impairment of the functions of the liver. During the febrile stage of malaria artificially induced in three cases, the albumin fell in all three to 3.3 g./100 ml. or less. The α_2-globulin also fell to about three-quarters of its previous level and the γ-globulin rose (Guttman *et al.*, 1945). The reduction of the α_2-globulin may have been due to the reduction of cholesterol that occurred in these sera during the attacks. Dole and Emerson (1945) studied the proteins in the serum of eight patients suffering from relapsing malaria in which *P. vivax* was found in the blood. The relation of the time of taking the blood to the attacks is not reported. The concentration of albumin was somewhat low,

and the α_1- and γ-globulin moderately raised in three out of six cases. In a patient who had suffered from severe malaria due to *P. falciparum* for three weeks the concentrations of total protein and albumin were very low (3.64 and 1.5 g./100 ml.), the α_1-globulin was normal and α_2-, β-, and γ-globulin reduced.

In glandular fever Cohn and Lidman (1946) found that the albumin was not changed. The α_2-globulin was moderately raised in four out of seven cases, and the γ-globulin was raised in all and over double the normal in one case. As bilirubin was detected in the urine of all these patients and the amount of bromsulphthalein retained in the blood after 30 minutes was abnormally high,

In the sera of three cases of advanced leprosy examined by Seibert and Nelson (1943) the α-globulin was moderately raised and the γ-globulin raised to 2.0, 2.8, and 3.45 g./100 ml.

Benditt and Walker (1948) examined the sera of syphilitic patients. Out of 15 cases, at all stages, the concentration of albumin was at the lower limit of normal or below normal in all but two. Of the globulin fractions, α_1-globulin was little changed except in one case, α_2-globulin was slightly raised, β-globulin was normal, and γ-globulin was above the normal means given by these authors in all cases. However, none of the globulin fractions were above the normal range given by Seibert *et al.*, except that the γ-globulin was 2.09 g./100 ml.

TABLE X

MEAN CONCENTRATIONS OF PROTEINS (g./100 ml.) IN SERA OF PATIENTS WITH TUBERCULOSIS AND OTHER DISEASES (SEIBERT *ET AL.*, 1947)

	Number	Polysaccharide (mg./100 ml.)	Total Protein	Albumin	Globulin			
					α_1	α_2	β	γ
Normal ...	43	103	7.29	3.88	0.58	0.76	1.01	1.05
Tuberculosis (a)	20	109	7.27	3.62	0.56	0.78	1.00	1.30
Tuberculosis (b)	22	136	7.67	3.32	0.64	0.92	1.15	1.65
Tuberculosis (c)	26	159	7.63	2.69	0.78	1.21	1.19	1.76
Tuberculosis (d)	21	109	7.17	3.71	0.59	0.81	1.00	1.06
Tuberculosis (e)	13	112	7.20	3.54	0.62	0.84	1.11	1.08
Sarcoidosis ...	11	128	7.88	3.10	0.61	0.85	1.26	2.06
Carcinoma ...	23	145	6.66	2.80	0.66	1.09	1.09	1.03
Diabetes ...	6	115	7.16	3.65	0.50	0.81	1.23	0.98
Diabetes with tuberculosis	6	166	6.82	2.11	0.74	1.03	1.33	1.62

(a) Active minimal pulmonary lesions. (b) Active, moderately advanced pulmonary tuberculosis. (c) Active, far advanced pulmonary tuberculosis. (d) Minimal pulmonary tuberculosis of questionable clinical significance. (e) Moderately advanced pulmonary tuberculosis of questionable clinical significance.

it may be inferred that the functions of their livers were impaired.

Electrophoretic Patterns in Chronic Infections. —Seibert *et al.* (1942, 1949) have made thorough studies of the relation of changes in serum proteins to the stage of tuberculosis (Table X). They found a wide spread in the values found both in normal subjects and in patients. The albumin might be as low as 3.4 g./100 ml. in normal subjects; in the severest group, c (Table X), it ranged from 1.6 to 3.4. The range of γ-globulin in normals was 0.59 to 1.46 g./100 ml.; in Group c from 0.97 to 3.28. Seibert *et al.* (1947) discussed the significance of the deviations from the standard means. The variations from the normal of the mean concentrations of α_1-globulin, γ-globulin, and of albumin and α_2-globulin particularly in Group c were highly significant.

in one primary case and 1.47 and 1.80 in two secondary cases. Cooper, Craig, and Beard (1946) found rather larger changes in the γ- and α_1-globulins with no significant change in the α_2- and β-globulins. Cooper (1945) found the mean γ-globulin was doubled and the α- and β-globulins nearly doubled.

The great increase of total globulin in the serum of patients with lymphogranuloma venereum has been studied extensively by precipitation methods, but few electrophoretic studies have been made. Kabat, Moore, and Landow (1942) found that the γ-globulin made up 34 and 38% of the total serum protein in two cases. In one case mentioned by Gutman (1948) the concentration of albumin was 3.25 and that of γ-globulin, 5.1 g./100 ml.

A number of investigators have found that the total protein and globulin are very high in the

serum of kala azar patients and fall as the condition improves. Cooper, Rein, and Beard (1946) examined the sera of two patients. In the serum of one, who was clinically ill, the total protein was 12.1 g./100 ml., the γ-globulin 7.76, and the albumin 2.62 g./100 ml. In the other case the total protein had been 8.9 g./100 ml. with 4.3 g./100 ml. of γ-globulin. After improvement the γ-globulin was still 2.46, although the albumin had gone up to 4.35. In this disease, even more than in malaria and glandular fever, it is probable that the changes in the serum proteins are due both to infection and to lesions of the liver. In the first serum examined by Cooper *et al.* and in our case (Fig. 4.H) the mobility of the γ-globulin was slow. Cooper *et al.* consider that this γ-globulin may differ from that found in excess in the serum in other infections and in diseases of the liver. Their serum did not give a positive formol-gel test, but the serum of our case (Fig. 4.H) gave the strongly positive reaction usual in kala azar sera.

Striking changes are found in some diseases of obscure origin which may be regarded as secondary responses to some chronic infection. In sarcoidosis, proved by biopsy, Fisher and Davis (1942) found that the albumin was low in active cases and the γ-globulin over 2.0 and might rise to 3.8 g./100 ml. In the 11 cases studied by Seibert *et al.*, included in Table X, the albumin was low. The α-globulins were, at most, little raised in sarcoidosis whereas the average γ-globulin was higher than in active, far advanced pulmonary tuberculosis. The small and scattered sarcoid lesions found in the liver (Scadding and Sherlock, 1948) are not likely to affect the electrophoretic pattern. Seibert *et al.* (1947) suggest that the difference in electrophoretic pattern may be of value in diagnosis. But it is rather doubtful whether the pattern would be of much use owing to the wide spread of the values found in the two diseases.

In two sera from cases of lupus erythematosus, in which the total globulin was high, Coburn and Moore (1943) found that 22 and 46% of the protein was γ-globulin. The albumin was low and the α- and β-globulins normal. They found that the γ-globulin might still be high after clinical recovery. This may be due to damage to the liver as there may be some infiltration and increase of fibrous tissue in the liver.

Lovgren (1945) reported the distribution in the serum of patients with rheumatoid arthritis. In some of these the γ-globulin was over 33% of the total protein. In most of the cases the albumin was little reduced and the α- and β-globulins were normal. Malmros and Blix (1946) included seven

cases diagnosed as rheumatoid arthritis. In three of these the γ-globulin was 3.0, 3.1, and 3.1 g./100 ml. respectively. One of these patient's had acute myocarditis and pleurisy, and a second an endocrine disorder. Dole and Rothbard (1947) followed up a case for eight months during which time the patient's condition improved considerably. The albumin was moderately reduced. Absolute concentrations of α_1-, α_2-, and γ-globulins were raised compared with Dole's normals (γ-globulin to about 1.5 g./100 ml.), but the albumin rose and the α_2-globulin fell to normal at the end of eight months. The α_1-globulin and γ-globulin remained high. In one serum, which we examined because it gave a strong thymol-turbidity reaction, the concentrations were albumin 3.2, α-globulin 1.0, β-globulin 1.1, and γ-globulin 3.9 g./100 ml.

Malmros and Blix (1946) examined the sera of nine cases of erythema nodosum. Two were classed as tuberculous and the remaining seven as rheumatic. The albumin was low in all. In four cases the γ-globulin was over 2, and in seven the α-globulin was over 0.7 g./100 ml.; maximum figures, γ-globulin 4.0, and α-globulin 1.3 g./100 ml.

The occurrence of an increase of α-globulin in the early stages of acute infections and in the more active stages of chronic infections suggests that this increase is a response to the destruction of tissue. Cuthbertson and Tompsett (1935) found that the serum globulin rose to high levels after fractures of bones of the leg. The highest figures were found in the first week after the injury. At the same time the albumin fell. In one case manipulation under an anaesthetic was followed by an increase of the globulin to 9 g./100 ml. Shedlovsky and Scudder (1942) found no increase of globulin in the serum of one patient three days after his tibia and fibula were broken. In another case the total globulin was 3.0 g./100 ml. two days after fracture of a femur, and both α- and β-globulin were moderately high. On the other hand Chambers and Gjessing (1946) found that the α_1- and α_2-globulin in the serum of a dog were doubled in the first week after one tibia had been broken under an anaesthetic. Burns, subcutaneous injection of turpentine to produce a sterile abscess, and injection of bis (β-chloroethyl) sulphide (Gjessing and Chambers, 1946) were all followed by an increase of α_1- and α_2-globulin. Perlmann, Glenn, and Kauffman (1943) did not find a significant change in the α-globulin in the serum of calves that had been severely burned. We (May and Hoch, 1949) examined the serum of a patient before and after his spleen was irradiated. Although the size of the spleen was very much

reduced in a few days, the serum proteins did not change except for a slight rise of the α-globulin (Fig. 4). Peterman, Karnofsky, and Hogness (1949) found no significant change in the electrophoretic patterns of the serum of patients with Hodgkin's disease and leukaemia after treatment with nitrogen mustards, methyl-bis (β-chloroethyl) amine hydrochloride and tris (β-chloroethyl) amine hydrochloride, although these reagents destroy lymphoid and haematopoietic tissue and, occasionally, tumour tissue. In view of the changes found in various other diseases not necessarily associated with destruction of tissue, it is doubtful whether an increase of α-globulin can be regarded as evidence of any special process.

Protein Patterns by Precipitation Methods.— The maximum amount by which concentrations of the α_1- and α_2-globulins increase are of the order of 0.4 and 0.8 g./100 ml., and some of the α-globulin is left in solution with albumin. Increase of the α-globulins, alone, will not produce a great increase in the total globulin. Since the albumin concentration is almost always reduced, increase of the α-globulin, alone, will not produce an increase of the total protein. An increase of globulin is mainly due to increase of γ-globulin. A great increase of globulin and an increase of total proteins are evidence of an increase of γ-globulin, with or without increase of α-globulin.

Although the albumin estimated by the usual precipitation methods, such as by half saturation with ammonium sulphate or by Howe's method, exceeds that estimated by electrophoresis, the two estimates usually vary in the same direction. Thus Dole (1944) found that the albumin/globulin ratio found by Howe's method was about 1.5 times that found by electrophoresis both in normal sera and in sera from ill persons, including that of the typhus fever patient he studied.

It is usually found that the total globulin, estimated by salting out methods, rises during acute infection. Malmros and Blix (1946) used both salting out and electrophoresis in 25 sera of patients with rheumatic fever and tonsillitis. In five cases the increase shown by electrophoresis was missed by the salting out method. Moen and Reimann (1933) found that the globulin (Howe's method) of the serum of pneumonia patients might be appreciably raised on the second day of fever; but the highest figure found was on the tenth day of the disease. The albumin fell in all cases, so that the total protein was low. On the thirtieth and forty-eighth days, during convalescence, the globulin might still be over 3 g./100 ml. and the

albumin still low. Stacey (1947) also found this persistence of an increase in the globulin after the acute stage. The globulin in the serum of a patient who had had an appendix abscess was still high after the appendix had been drained for 20 days.

As a patient's clinical condition improves, the albumin rises and the globulin falls. Thus the albumin rose from 3.9 to 5.6 and the globulin fell from 7.2 to 2.6 g./100 ml. in the serum of a tuberculous patient as the condition improved, although, in another case, the albumin fell from 5.4 to 5.1, with a coincident fall of globulin from 3.4 to 2.6 when the patient improved and gained 15.5 lb. in weight (Eichelberger and McCluskey, 1927). In rheumatoid arthritis Davis (1935–6) found that the globulin (Howe's method) fell from 3.9 to 3.0 with improvement in a severe case and rose again to 4.0 as the patient relapsed, and in another less severe case fell from 3.1 to 2.0 when the patient recovered. However, in another severe case the globulin, as well as the albumin, rose although the patient felt better and had gained 7 lb. in weight.

When patients with lymphogranuloma venereum were treated with sulphanilamide the albumin rose and the globulin fell; thus in one case the concentrations of proteins before and on the seventh and 212th days after treatment began were: Albumin, 1.86, 3.37, 3.75, and globulin, 5.87, 4.28, 2.60 g./100 ml. (Schamberg, 1941).

In those conditions in which the increase of γ-globulin is highest, namely, typhus fever (Tierney and Yeomans, 1946), lymphogranuloma venereum (Schamberg, 1941), leprosy (Frazier and Wu, 1925), sarcoidosis (Fisher and Davies, 1942), kala azar (Ling, 1930), and some cases of rheumatoid arthritis (Davis, 1935–6), the total globulin, measured by salting out methods is very much increased. In typhus fever the globulin may be high on the fifth day after onset, before the rash appears and before the Weil–Felix reaction becomes positive (Tierney and Yeomans, 1946); it rises to high levels (over 4 g./100 ml.) and remains high for five weeks after the temperature has fallen. These changes are comparable to the changes of γ-globulin found by Dole, Yeomans, and Tierney (1947).

The γ-globulin is mainly precipitated in Howe's euglobulin and pseudo-globulin I; these two fractions may be taken together. The α-globulins are included in the Howe's pseudo-globulin II and albumin fractions. (The normal distribution found by Howe's method is given in Table II.) Moen and Reimann (1933) found that the pseudo-

globulin II was raised in some cases of pneumonia in early stages, but the eu- + pseudo-globulin I was also raised and this fraction alone was raised in the later stages. In the subacute and chronic infective diseases the increase is almost always confined to the eu- + pseudo-globulin I fraction. Gutman and colleagues (1941) present a table that includes cases of lymphogranuloma venereum, sarcoid, lupus erythematosus, leprosy, and tuberculous adenitis in which this fraction was increased. Other examples are kala azar (Ling, 1930), and lupus erythematosus (Coburn and Moore, 1943). In rheumatoid arthritis the main change is in this fraction although the pseudo-globulin II may also be increased to an average level of 0.68 g./100 ml. in severe cases (Davis, 1935–6).

Other Protein Changes.—A protein, the C-reactive protein, has been detected in the serum of patients suffering from acute infectious diseases, and has the characteristic of forming a precipitate with the somatic C-polysaccharide of pneumococci. The reaction differs from an ordinary precipitin reaction in that no precipitate is formed unless calcium ions are present in the solution (Abernethy and Avery, 1941). The presence of this protein in the serum is not characteristic of any particular infection. Strong reactions with the polysaccharide have been found in the serum of patients suffering from lobar pneumonia, other infections of the respiratory tract, miscellaneous purulent infections, septicaemia, Still's disease, typhoid fever, coli pyelitis, rheumatic fever, and tuberculosis (Ash, 1933). This protein was found in the sera of patients with rheumatoid arthritis by Dole, and Rothbard (1947) and Wallis (1946) have reported finding it in other cases. The reactions that Wallis found were weak. We examined a number of sera and found only one that reacted strongly with the polysaccharide. The C-reactive protein is precipitated by ammonium sulphate between 50 and 75 saturation; it appears to be an α_1-globulin (Perlmann *et al.*, 1943). It has been crystallized (McCarty, 1947). As an antigen it is different from other serum proteins. The amount of precipitate formed on mixing sera of patients with antisera to the C-reactive protein, suggests that the amount in these sera may be of the order of 0.1 g./100 ml. It contributes towards the increase of α_1-globulin found in acute infections, just as the true antibodies contribute to the increase of the γ-globulin.

Blix, Tiselius, and Svensson (1941) noted the large amount of polysaccharide in the protein fractions of the serum of a patient with pneumonia,

Table V. The largest amount was in the α-globulin. Seibert *et al.* in their analysis on the sera of patients with tuberculosis and carcinoma found that the polysaccharide content of the serum rose with the α_2-globulin and was not correlated with γ-globulin and albumin.

Cartwright and Wintrobe (1949) have found that the iron-binding capacity of serum, which is a measure of the concentration of the metal-binding protein, may be low or very low in the serum of patients suffering from infections and rise rapidly on recovery. The iron-binding capacity of the serum falls on the day of injection of typhoid vaccine and rises again on the following day. This reduction of iron-binding capacity is not the cause of the low concentration of serum iron and the anaemia of infections, as the metal-binding protein was less saturated than it normally is.

Effects of Deficiency of Protein.—Ancel Keys *et al.* (1946) found that the average serum proteins of a group of young men who lived for months on a diet that supplied little protein and insufficient calories fell by only 0.73 g./100 ml. The electrophoretic patterns were practically unchanged. These subjects lost much of their body protein and became oedematous, with a great increase in the extracellular water. Their condition resembled that found in famine oedema. In a large number of cases of famine oedema the serum albumin is much reduced.[*] However, in recent investigations, for example, Sinclair (1948) it has been found that the serum albumin is about normal in a large proportion of cases of famine oedema. It seems that oedema may be due to some additional cause as well as to the reduction of the serum albumin, and that some other factor besides loss of body proteins is involved when the albumin in the serum is reduced. Rytand (1942) studied a patient who had had recurrent oedema with low serum proteins for some years. There were no albumin or casts in the urine. The serum protein varied between 3.0 and 4.7 g./100 ml. The electrophoretic pattern was: albumin 2.83, α-globulin 0.27, β-globulin 0.70, and γ-globulin 0.69 g./100 ml. In this case the diet was adequate and the defects seems to have lain in the synthesis of serum protein. In another case of recurrent oedema (Schick and Greenbaum, 1945), when the serum albumin was 3.3 and the globulin 0.8 g./100 ml., no peak corresponding to γ-globulin was

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[*]A list of investigations is given in *Recommendations with regard to Methods of Investigation of Nutrition*, (1945), issued by the Nutrition Society.

seen. In this case the Schick and Dick tests were negative, so that the serum contained some anti-bodies, but the association of antibodies with γ-globulin was shown, as no agglutinins to typhoid bacilli and whooping-cough antibodies were formed in response to injection of vaccines. In a third case, a girl whose diet had been poor, the albumin was 2.0 and the globulin 1.1 g./100 ml. when the patient was first seen (Krebs, 1946). Later the electrophoretic pattern was: albumin 1.81, α-globulin 0.49, β-globulin 0.46, and γ-globulin 0.16. After two injections of typhoid vaccine no agglutinins were detected. The patient was given a high protein diet, and the albumin and γ-globulin rose to 2.54 and 0.68 g./100 ml. Deficiency of protein may therefore in some cases lead to reduction of γ-globulin as well as to reduction of albumin. For other examples see cases F and G (Fig. 4).

The great reduction of protein in the serum of patients with kidney disease and gross oedema was noted by Bright. Since Epstein (1917) attributed the gross oedema of nephrosis to reduction of the osmotic pressure of the serum proteins a very large number of observations have been made. These have confirmed the close association of gross oedema with reduction of the serum albumin, although other factors are certainly involved in causing oedema. The electrophoretic patterns found will be discussed in the section dealing with lipaemia. It has been suggested that the serum proteins in this condition may be abnormal. These apparent abnormalities are due to the use of impure fractions for study. Alving and Mirsky (1936) found in the plasma of nephrotic patients that the albumin fraction, that is, the protein not precipitated by half saturation with ammonium sulphate, had an abnormally low content of cystine. However, Brand et al. (quoted by Edsall, 1947) have found that certain α-globulins contain relatively little cystine. α-globulin makes up a relatively large proportion of the so-called albumin fraction, when the albumin concentration is low, and this α-globulin accounts for these low cystine contents. The results obtained by Goettsch and Reeves (1936) and Goettsch and Lyttle (1940) using immunological methods can be explained by a quantitative change in the serum protein fractions. The preparations used by Bourdillon (1939) for estimating the osmotic pressure of the albumin in the serum of nephrotic patients, certainly contained globulin which accounts for the low osmotic pressures. Other investigations (Hewitt, 1927, 1929: Widdowson, 1933) have detected no difference between the albumin of serum and urine.

Deficiency of protein is probably one of the factors that cause reduction of the concentration of serum proteins and changes in the electrophoretic patterns in a number of diseases. Seibert et al. (1947) found that the mean concentration of the serum albumin of 23 patients with carcinoma was 2.8 g./100 ml. The α_2-globulin was moderately raised; this might be in response to destruction of tissue. If cases which had metastases in the liver with jaundice were excluded, the mean γ-globulin was low. In most cases the γ-globulin peak was much flattened. Low serum albumin was found in two out of three cases of carcinoma (without secondaries in the liver) by Luetscher (1941), and in one of these the γ-globulin was low.

The albumin is particularly low in the serum of patients with carcinoma of the stomach (Abels et al., 1942). Peterman and Hogness (1948) examining the serum of 25 patients with carcinoma of the stomach found that the albumin was under 3 g./100 ml. in all of them. The α_1-globulin was in the upper range or above the range of normal found by these authors, but little raised as compared with the normals of Seibert et al. The α_2-globulin was slightly raised, but the γ-globulin was not low either in cases without or in cases with metastases. In these cases the α_1-globulin fell on treatment with protein hydrolysates, but the albumin did not rise although the nitrogen balance was positive. Even after the primary tumours had been removed and the patients, with no obvious metastases, had eaten a normal diet for two months, the serum albumin was still under 3.6 g./100 ml. Peterman and Hogness also found that the serum albumin was low in six cases of gastric ulcer.

Lipaemia.—The proteins to which lipids are attached are in the α- and β-globulins. It is therefore to be expected that when the amount of lipid in the serum is raised the α- and β-globulins will be raised. Lewis, Schneider, and McCullagh (1944) found the β-globulin high in untreated diabetes mellitus, a condition in which the lipids of the plasma are high, but on treatment the β-globulin fell to normal. Longsworth et al. (1945) point out that the sum of α- and β-globulin is higher than normal in the plasma of women immediately after childbirth and that in the plasma of blood taken from the umbilical cord this sum is lower than normal; the concentration of total lipids of plasma at the end of pregnancy is nearly 150% and in cord blood only about 30% of the concentration in the plasma of normal adults. In hypothyroidism the cholesterol in the

plasma is increased and the β-globulin is high (Lewis and McCullagh, 1944).

In their early studies Longsworth *et al.* (1939) found that not only was the concentration of albumin in serum in nephrosis even lower than had been found by salt fractionation, but also that the concentrations of α- and β-globulins were increased. These observations have been confirmed by Longsworth and McInnes (1940), Luetscher (1940), and Malmros and Blix (1946). Thorn *et al.* (1945) made their electrophoresis runs at *p*H 8.6 and found that the α_1-globulin and γ-globulin were low. The increase of absolute concentrations of α_2- and β-globulin are less striking than those of the relative concentrations. Thus in the serum of a patient with gross oedema

may be little raised although the total protein is low and the concentration of cholesterol in the serum is moderately raised. These patients are continually losing protein in the urine in amounts up to 20 g. and more daily. If they lose their oedema they are found to be much wasted. It is reasonable to suppose that the changes found in the serum are a combination of changes due to loss of protein and to lipaemia, and are not a change peculiar to nephrosis. Extreme changes may be found as soon as one month after the appearance of oedema, as in case W.H. of Table XI.

As abnormally large amounts of lipids are associated with protein in the complexes that give rise to the α- and β-peaks, the globulin is overestimated

TABLE XI

RELATION BETWEEN OEDEMA, CHOLESTEROL, AND PROTEIN COMPONENTS (g./100 ml.) IN NEPHROTIC PLASMA
(THORN *et al.*, 1945)

Case	Oedema	Cholesterol (g./100 ml.)	Total Protein (g./100 ml.)	Albumin		α_1		α_2		β		φ		γ	
				R*	A†	R	A	R	A	R	A	R	A	R	A
J.R.	++++	1.10	2.8	7	0.19	4	0.11	42	1.18	28	0.78	16	0.45	3	0.08
L.I.	+++	1.17	3.8	17	0.65	5	0.19	36	1.37	22	0.84	16	0.62	4	0.15
W.H.	+++	0.755	4.1	17	0.70	5	0.205	21	0.86	38	1.56	15	0.62	4	0.16
P.S.	+++	1.00	5.3	26	1.38	8	0.42	22	1.16	30	1.59	9	0.47	5	0.26
K.N.	+.	0.43	5.6	37	2.06	6	0.33	15	0.84	22	1.23	12	0.67	8	0.45
								R	A						
E.B.	+	0.88	4.1	46	1.88	4	0.16	34	1.4			13	0.53	3	0.12
R.S. Serum	±.	0.34	5.6	32	1.79	5	0.28	20	1.12	23	1.28			20	1.12

R* Relative concentration as percentage of total protein. A† Concentration in g./100 ml.

the α_2-globulin made up 42% and the β-globulin 28% of the total protein, but the absolute concentration of α-globulin was only twice the normal and that of β-globulin was under the average normal.

The concentrations of cholesterol, phosphatides, and neutral fat in the serum are much increased in nephrosis (see case illustrated in Fig. 4J.), but the concentration of any lipids in sera which have been examined by electrophoresis have rarely been reported. The relation to concentration of cholesterol is well illustrated by the data given in Table XI. Longsworth and MacInnes (1940) and later Blix and Pedersen (1947) have shown that the α- and β-globulins of nephrotic sera are much reduced by extraction of the lipids ; extraction of normal sera has little effect. Longsworth and McInnes found that the concentration of β-globulin in a nephrotic serum, after extraction with ether, was below the normal average. As the case illustrated in Fig. 4A shows, the α- and β-globulin

when concentrations are calculated from the electrophoretic pattern, and, in consequence, the albumin is underestimated. Also albumin makes up an abnormally low proportion of the protein left in solution after precipitation by half-saturation with ammonium sulphate or by Howe's method. The discrepancy between the amounts of albumin found by electrophoresis and by salt fractionation is much greater in nephrotic sera than in other sera. However, neither albumin is the true albumin, in the sense of being free from associated globulin or lipid.

In the urine the albumin, as it has the lowest molecular weight, makes up a very much larger proportion of the total protein than in the serum. However, Luetscher (1940) found a considerable amount of γ-globulin in the urine of a patient with amyloid disease. From the figures of Longsworth and MacInnes (1940) it appears that the concentration of albumin in the urine is higher than in the plasma ; this is probably due to underestima-

tion of the concentration of albumin in the plasma.

In other types of nephritis, not characterized by gross oedema and lipaemia, the serum proteins differ little from normal, the only change being a reduction of albumin.

Liver Disease.—The change of serum proteins characteristic of disease of the liver parenchyma is an increase of the γ-globulin. The albumin is reduced, sometimes to very low levels, and the other fractions are usually little changed. These changes, first detected by Luetscher (1940) have been reported by numerous observers (for example, Gray and Barron, 1943 ; Thorn, Armstrong, and Davenport, 1946) since. The relative concentration of γ-globulin is always raised ; the absolute concentration is usually near 2 g./100 ml., and may rise to 4 g./100 ml., but is sometimes little above normal (Thorn, Armstrong, and Davenport, 1946). Sherlock (1946) examined cirrhotic livers by biopsy ; she classed as " latent " those in which cell damage was minimal and bands of mature fibrous tissue disrupted the normal architecture and as " active " those with retrogressive parenchymatous changes and regeneration of surviving cells. The average concentrations of serum albumin and globulin in the active cases were 2.8 and 4.0 g./100 ml., and in the latent cases 4.2 and 2.5 g./100 ml. Cases in which the γ-globulin is little raised are probably latent.

With local lesions of the liver, such as metastases of carcinoma, the γ-globulin may be normal or may be raised to 2.4 g./100 ml. (Gray and Barron, 1943). Obstruction of the bile ducts, unless associated with metastases in the liver or a secondary hepatitis, causes no significant change of serum proteins, or a rise of β-globulin due to increase of cholesterol in the serum.

In infective hepatitis Martin (1946) found that the relative concentration of γ-globulin was increased. The relative and absolute concentrations of albumin were moderately reduced in the early stages. The increases of absolute concentrations of γ-globulin were slight in five of the six cases in which absolute concentrations could be calculated. In the sixth the concentration was 1.76 g./100 ml. on the twenty-third day from the onset of symptoms. In this case excess of bromsulphthalein was retained 57 days after the onset and the γ-globulin was then 1.94 g./100 ml. In six cases of acute hepatitis (one arsenical jaundice and five catarrhal jaundice) Gray and Barron (1943) found γ-globulin concentrations of 2.85 and 3.0 g./100 ml. in two cases ; in three others also the γ-globulin was above the normal range. The

β-globulin was over 2 g./100 ml. in two cases (including the arsenical jaundice) but was not raised in the other cases.

Thorn, Armstrong, and Davenport (1946), using Howe's method, did not find the usual discrepancy between the amount of albumin estimated by salt fractionation and by electrophoresis. However, Moore et al. (1941) found as big a discrepancy in the serum of a hepatitis patient as in normal serum. In the serum of subjects intentionally infected with hepatitis, albumin estimated by these methods fell to a minimum and globulin rose to a maximum in the second week after the onset of symptoms. The albumin returned to its original level at the end of a month, but the globulin was still raised at the end of 60 days (Havens and Williams, 1948). The increase in the globulin is mainly accounted for by a rise in Milne's fraction which corresponds to γ-globulin. As the albumin returned to normal while the globulin remained high the total serum proteins were increased at five to six weeks after onset. Rennie (1945) found that the globulin might continue to rise for some months after the onset of acute hepatitis. In chronic hepatitis and cirrhosis of the liver the ratio of albumin to globulin is correlated with activity of the Kupffer cells and damage to cells of the liver parenchyma (Franklin et al., 1948). Post and Patek (1942) found that patients with liver disease were more likely to recover when their serum albumin was above 3.5 g./100 ml. Higgins and his colleagues (1944) found that 20 out of 24 patients who had recovered from hepatitis had serum albumin over 3 g./100 ml. Sherlock (1946) using biopsy studies considered that the serum albumin fell and globulin rose as the severity of the liver lesions of patients with acute hepatitis increased. She also found that albumin fell with the duration of obstructive jaundice and was not more than 3 g./100 ml., in 60% of cases, whereas the globulin changed but little.

Myelomatosis.—Bence-Jones protein, which is excreted in the urine by about half the patients suffering from multiple myeloma, has a molecular weight of 37,000 (Svedberg and Pedersen, 1940) ; this is just over half the molecular weight of serum albumin. Its mobility in phosphate buffer (pH 7.4, ionic strength 0.2) ranges from 1.4 to 3.1 ; these values lie between the mobilities of β- and γ-globulin or are equal to that of β-globulin. Gutman et al. (1941) found that in most cases precipitation by ammonium sulphate began at 40% and was complete at 50 or a little over 50% saturation. Most of the Bence-Jones protein was not usually precipitated from urine by 21.5% sodium

sulphate unless the urine was made strongly acid. Antigenically, Bence-Jones proteins differ from normal serum proteins. Two types, differing antigenically, have been distinguished, but may occur together in the same urine (Hektoen and Welker, 1940).

On heating at neutral or slightly acid reaction Bence-Jones proteins coagulate at 45 to 58° C. In many cases the coagulum redissolves on boiling. The resolution is promoted by the presence of salt, salts of divalent kations being more effective than those of univalent kations. The protein is reprecipitated on cooling. In other cases the coagulum does not redissolve on boiling but forms a dense curd which floats in the froth on the surface.

Gutman and his colleagues (Gutman *et al.*, 1941 ; Moore *et al.*, 1943 ; Gutman, 1948) have made a thorough study of the electrophoretic components of the serum of myelomatosis patients, and of the relation of these components to the Bence-Jones protein in the urine and the fractions found by Howe's method. They classed the sera in three groups: (1) Sera in which protein of the mobility of γ-globulin is increased and no Bence-Jones protein is detected by electrophoresis or ultracentrifugation (cf. Fig. 4D). (2) Sera in which various anomalous patterns are formed, usually with extra components of mobility equal to that of β-globulin or lying between those of β- and γ-globulin ; in some cases the extra component is Bence-Jones protein. (3) Sera with apparently normal patterns. In groups (1) and (2) the concentration of albumin is low and often very low. Moore, Kabat, and Gutman (1943) found that the α-globulin was increased in two cases. The sera studied by Longsworth, Shedlovsky, and McInnes (1939), Kekwick (1940), Blackman *et al.* (1944), Malmros and Blix (1946) and Martin (1947) fit into these groups. In one of the cases of Malmros and Blix the serum pattern was essentially normal at the first examination ; a year later the albumin was low and both β- and γ-globulin slightly increased. In one case studied by Moore, Kabat, and Gutman (1943) a very large β-component was found on one occasion, and on another a component of mobility between those of β- and γ-globulin. Gutman *et al.* (1941) showed that the patterns found in Groups (1) and (2) could be reproduced by adding urinary Bence-Jones protein to normal serum. However, in most of the investigations made with the ultracentrifuge (McFarlane, 1935 ; Jersild and Pedersen, 1938 ; von Bonsdorff *et al.*, 1938 ; Kekwick, 1943) no protein with the low sedimentation constant of Bence-Jones protein has been found in serum, but Packalén (1939) found a protein of low molecular weight which crystallized. Moore, Kabat, and Gutman (1943) found components with low sedimentation constants in two sera ; in one the mobility of the abnormal component was equal to that of β-globulin and in the other lay between those of β- and γ-globulin. These components, therefore, had the physical properties of Bence-Jones proteins. However, in two other sera in which the abnormal component had a mobility between those of β- and γ-globulin their sedimentation constants were equal to that of normal γ-globulin. In one of Kekwick's (1940)' cases three abnormal components were found which had sedimentation constants above that of the bulk of the normal globulin. Abnormal components are therefore not usually identical with Bence-Jones protein. In a case of Moore *et al.*, in which the abnormal component had a low sedimentation constant and mobility between those of β- and γ-globulin, this component was antigenically identical with the Bence-Jones protein of the urine. In another serum which contained a large amount of protein resembling normal γ-globulin in mobility and sedimentation constant, a small amount of Bence-Jones protein was detected immunologically.

Bence-Jones protein may be present in or absent from the urine in cases of all three groups. It was found in the urine in two of the three cases of Moore *et al.*, in which its presence in the serum was established. Malmros and Blix (1946) found that on one occasion a patient's urine contained only traces of protein ; a year later it contained 1.5% of Bence-Jones protein. On both occasions the serum contained a large amount of protein of mobility equal to that of β-globulin.

The same type of electrophoretic pattern may be found in the serum of patients with single tumours, multiple tumours, or generalized osteoporosis.

The total protein concentration in serum may be very high. In just over half of the 43 cases quoted by Gutman (1948) the concentration was over 8 g./100 ml. and in five it lay between 12 and 13.7 g./100 ml. In infective conditions, as already noted, any increase of the total globulin is usually accounted for by an increase of both Howe's euglobulin and pseudoglobulin I fractions. In myelomatosis the euglobulin fraction may remain low although the globulin is much increased ; this may be found with sera of Groups (1) and (2). On the other hand the euglobulin fraction may be much increased in sera of both these groups. As Bence-Jones protein is but partially precipitated

by 21.5% sodium sulphate, the concentration of albumin in sera that contain Bence-Jones protein, when estimated by Howes' method, may be much higher than that found by electrophoresis.

Part of the calcium in serum is combined in an indiffusible form with protein. This fraction varies with the concentration of albumin. As globulin binds relatively little calcium, the concentration of bound calcium in the serum of myeloma patients may be little increased (Gutman and Gutman, 1937) although the total protein is high. In some cases high concentrations of calcium are found in the serum. Laake (1949) ascribes these high concentrations to the mobilization of calcium from the bones.

Miscellaneous Diseases.—Various diseases that do not fall into the preceding groups have been studied. These include leukaemia and aplastic anaemia (Longsworth et al., 1939); leukaemia, lymphosarcoma and Hodgkin's disease (Peterman, Karnosky, and Hogness (1948); hyper- and hypo-thyroidism (Lewis and McCullagh, 1944); Addison's disease (McCullagh and Lewis, 1945); Cushing's syndrome and acromegaly (Lewis and McCullagh, 1947). The albumin was usually low and the α-globulin slightly raised, the degree of change varying with the severity of the disease; with improvement of the condition the pattern reverted towards normal. In Addison's disease, the reduction of albumin was not in keeping with the severity of the disease. In one severe, untreated case the concentration was 4 g./100 ml., but in a moderate case it was 2.66, and in a mild case 3.3 g./100 ml. In eight cases of Cushing's syndrome the γ-globulin was low (Lewis and McCullagh, 1947). The increase of γ-globulin on treatment (for example, from 0.53 to 1.22 g./100 ml.) was more striking than the increase of albumin.

Other Abnormal Proteins.—The abnormal component in the serum of one of the cases in the series of Moore, Kabat, and Gutman (1943) had a mobility between those of β- and γ-globulin but was not Bence-Jones protein, and was studied further by Shapiro, Ross, and Moore (1943). It was a viscous protein with a molecular weight of about 160,000.

Lerner and Watson (1947) proposed that the name " cryoglobulin " should be used for a group of proteins that have the property of precipitating or gelifying from cooled serum. They quote four examples described by other investigators. Shapiro and Wertheimer (1946) described another example in which a crystalline protein was separated from

the serum of a patient with periarteritis nodosa. Four of the patients in the series quoted had multiple myeloma.

Lerner, Barnum, and Watson (1947) examined sera for the presence of cryoglobulin from patients with a number of diseases. They found that small precipitates, of the order of 10–20 mg./100 ml., formed on cooling the sera of patients with lymphatic leukaemia, congenital hypoprothrombinaemia, bronchopneumonia, bronchiectasis, rheumatic heart disease, and subacute bacterial endocarditis. About 800 mg. of cryoglobulin precipitated from 100 ml. of the serum of a patient with cardiovascular renal disease. This protein (Lerner and Greenberg, 1946) was electrophoretically homogeneous at pH 4.7; it was soluble at 2° C. in distilled water but almost completely insoluble in 0.5% solution of NaCl; its molecular weight was 190,000.

In McFarlane's (1935) studies of pathological sera there is no evidence of the presence of abnormal proteins except in those derived from patients with multiple myeloma. However, the components seemed to be less homogeneous than those of normal sera.

Peterman and Hogness (1948b) examined sera in acetate buffer at pH 4.0. Most of the serum protein is positively charged at this pH, but they detected a protein which was negatively charged and had therefore a low isolectric point. The sera from patients with carcinoma of the stomach, carcinoma of the lung, leukaemia, and Hodgkin's disease contained more of this protein than was found in the sera of normal persons or patients with miscellaneous cancer.

In their second account of the copper-sulphate method of estimating the specific gravity Phillips, Van Slyke, et al. (1945) reported that the correct formula for calculating protein concentration (P) from specific gravity (G) should be

$$P = 377 (G_p - 1.0070)$$

The values of P calculated with this formula are close to those calculated with the formula

$$P = 364 (G_p - 1.0060)$$

we recommended (Hoch and Marrack, 1945a), when the specific gravity is of the order of that found with normal sera. Phillips et al. found that comparisons of specific gravities with protein concentrations in sera from miscellaneous hospital patients measured by macro-Kjeldahl estimates give a mean value of 360 instead of 377 for the constant. This would imply that the relation of the density of serum to the concentration of protein therein was abnormal, owing either to the presence of abnormal proteins or to abnormal relative pro-

portions of the proteins. We found, however, that the values for the protein concentrations obtained by specific gravity methods, using our formula, agreed with those calculated from the Kjeldahl-N (x6.25) to within 4% with the sera of normal people, of those with malnutrition, and of women before and after childbirth (Hoch and Marrack, 1945a, b). The results of further experiments with nephritic sera and sera of patients with other diseases, including infective hepatitis, cirrhosis of the liver, coeliac disease, pernicious anaemia, and, albuminuria are shown in Fig. 5.
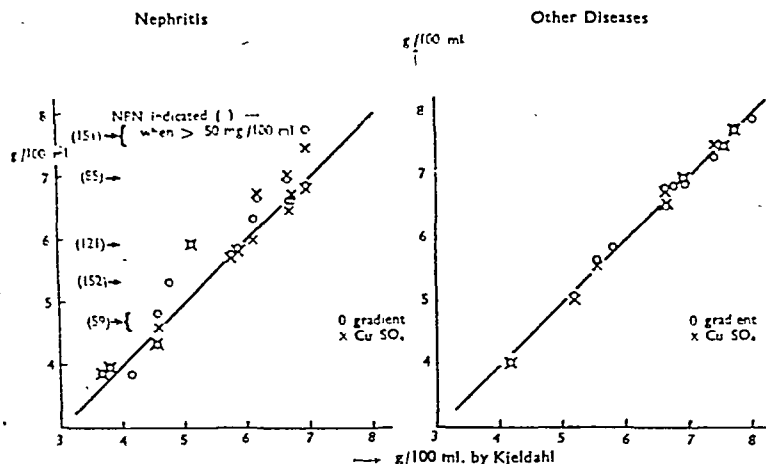
FIG. 5.—Relation of concentration of protein, calculated from the specific gravity, to the concentration calculated from the nitrogen content in pathological sera.

The errors ranged from +15.4 to −7.0% in nephritis, but they were within ±3% in all the other cases. There is therefore no evidence of an abnormal relation between specific gravity and protein concentration in these cases, and the same formula is applicable both to normal and to pathological sera.

VI. Flocculation Reactions

In considering the relation of various aggregation and flocculation reactions to the fractions of serum proteins it should be remembered that these fractions, whether separated by precipitation with salts or by electrophoresis, are not homogeneous, nor is their composition constant. The α-globulin of infectious conditions differs from that of nephrosis; and the γ-globulin of liver disease from that of myelomatosis. It is probable, however, that fibrinogen does not vary.

Most observers have found that electrophoretically isolated fractions produce effects similar to those in Table XII. There is some disagreement about the differences between the effects of fractions isolated from normal serum and from sera of patients with disease of the liver. Kabat *et al.* (1943) found that γ-globulin separated at pH 7.4 from normal and pathological sera (cirrhosis and hepatitis) did not differ in efficiency in flocculating either colloidal gold or cephalin-cholesterol. Moore *et al.* found both equally efficient in flocculating cephalin-cholesterol, and McLagan and Bunn found that in hepatitis γ-globulin was the more effective. On the other hand, Moore *et al.* (1945) found that hepatitis albumin separated at pH 7.4 was a less efficient inhibitor than normal albumin of the cephalin-cholesterol reaction. Kabat *et al.* found that albumin did not significantly inhibit this reaction. The differences between the effects of albumin may be due to differences in the amounts of some inhibiting constituent, other than albumin, in the preparations. Bernsohn and Borman (1947) found that a preparation of albumin which contains only 3% of α-globulin made by the low-temperature-low-salt-low-dielectric-constant method did not inhibit the colloidal gold reaction. It is remarkable that Guttman *et al.* (1945) found that the albumin isolated from the serum, taken during an attack of malaria, was more effective in inhibiting the cephalin-cholesterol reaction than was albumin isolated from serum taken before or after the attack. Bernsohn and Borman agreed with MacLagan and Bunn in finding that preparations containing β-globulin, without albumin, inhibited the colloidal gold. The effects of α- and β-fractions on the cephalin-cholesterol reaction may be due to their affinity for lipids.

The thymol-turbidity and thymol-flocculation reactions differ from the two other flocculation reactions in the importance of lipids. Phospholipids and cholesterol make up about one-third of the weight of the floccules formed with serum. The turbidity produced by isolated γ-globulin with the thymol reagent alone was very small. For this reason cephalin was added to the reagent used in the tests shown in Table XII. The main change produced in the electrophoretic patterns by floc-

TABLE XII

EFFECT OF PROTEINS, SEPARATED ELECTROPHORETICALLY AT pH 8.0, ON FLOCCULATION REACTIONS
(MACLAGAN AND BUNN, 1947)

Reaction	Flocculator	Inhibitor	Difference between Proteins of Normal (N) and Hepatitis (H) Sera
Thymol (cephalin) turbidity	γ-globulin	Normal albumin	H γ-globulin slightly more effective than N. H albumin does not inhibit
Thymol (cephalin) flocculation	H γ-globulin	Not tested	N γ globulin does not flocculate
Colloidal gold, pH 7.8	γ-globulin	Albumin, α- and β-globulin	H γ-globulin more effective than N.
Cephalin–cholesterol	γ-globulin H (α + β)-globulin*	Albumin	H γ globulin much more effective than N. N α- and β globulin do not flocculate
Takata-Ara	H γ-globulin H (α+β)-globulin*	H Albumin	

* Two fractions not separated.

culation with the thymol reagent is a reduction of the relative area of the β-globulin peak (Cohen and Thompson, 1947). It is probable that this reduction is due to removal of lipid which contributes to the area of this peak. Kunkel and Hoagland (1947) suggest that some γ-globulin may form a light combination with lipid and that this fraction migrates with β-globulin. We have confirmed (Marrack, Johns, and Hoch, 1949) by immunological methods that the bulk of the protein in the floccules is γ-globulin. The intensity of the turbidity depends on the amount of lipid in the serum. Popper and colleagues (1949) demonstrated that absorption of a large meal of fat increased the intensity considerably. Kunkel and Hoagland found that in patients with acute hepatitis the intensity of the reaction ran parallel to the alterations of serum lipids in the earlier stages and of the γ-globulin during convalescence.

The positive flocculations tests found in diseases other than hepatitis and cirrhosis of the liver, for example, malaria, glandular fever, rheumatoid arthritis, and heart failure (Carter and MacLagan, 1946), are all probably due to an increase of γ-globulin and a decrease of albumin, secondary in some cases to damage to the liver. The sera of multiple myeloma patients do not give positive thymol turbidity reactions (Kunkel and Hoagland, 1947). Other flocculation and precipitation reactions such as the cadmium-turbidity test (Wunderly

and Wuhrman, 1947) and the zinc-sulphate reaction of Kunkel (1947) depend on an increase of γ-globulin in serum. In the series of sera examined by Malmros and Blix (1946) the γ-globulin was over 2 g./100 ml. when the formol-gel reaction was positive.

Erythrocyte Sedimentation Rate.—Gray and Mitchell (1942) studied the effects of electrophoretically isolated fractions on the erythrocyte sedimentation rate. Fibrinogen increased the rate most, α- and β-globulin were less effective, and γ-globulin least. Albumin delayed sedimentation.* These four factors therefore affect the E.S.R., even if the efficiency of the globulin fractions does not change with disease. The rate is not necessarily correlated with the concentration of any one component. Thus Shedlovsky and Scudder (1942) found that in infective conditions the E.S.R. was more closely correlated with the concentration of α-globulin than it was with the concentration of fibrinogen. In the series reported by Malmros and Blix (1946) the E.S.R. was high in some cases of multiple myeloma in which the only abnormalities were reduction of the albumin and increase of γ-globulin. In these cases the γ-globulin was probably abnormal.

*According to the latest report (Surgenor, Strong, et al., 1948) fibrinogen is the most effective protein, and the next most effective a carbohydrate-rich α_2-globulin, which also binds bilirubin.

Discussion

The changes found in the electrophoretic patterns are not characteristic of any disease. The outstanding changes are reduction of albumin, an increase of γ-globulin or a moderate increase of α-globulin. These are found in a variety of conditions. The increase in the relative areas of the α- and β-globulin peaks in the patterns in nephrosis appears to be secondary to an increase of the lipids of the serum, an increase that can be better detected by simpler means. The reduction of γ-globulin which may have a better claim to be regarded as specific of nephrosis is found in other conditions associated with deficiency of protein.

Owing to the wide spread of the concentrations of the components of normal serum, a component may change considerably without passing beyond the normal range. Such changes will not be detected unless the patient's serum is examined repeatedly. The average change found in one disease may be greater than the average found in another, but in individual cases the difference may be in the opposite direction ; thus the highest concentration of γ-globulin found by Siebert *et al.* (1947) in tuberculosis was higher than any they found in sarcoidosis, in which high figures were more common.

The bulk fractions separated by electrophoresis or by any other method are complex mixtures of several small components. It cannot be expected that changes in these bulk fractions will measure or even detect changes in the various components. The maximum amount of an antibody found in human serum is about 1 mg./ml., and the standard deviation of γ-globulin, according to Siebert *et al.* (1947), is 2.2 mg./ml. It is only in sera of hyper-immunized animals that the amounts of antibody rise to several mg./ml., and are enough to show in the electrophoretic pattern, and when the antibody is precipitated by antigen, an appreciable reduction of a peak can be seen. Possibly the reduction of β-globulin which is often found in infections is due to reduction of the metal-combining protein which normally makes up about one-quarter of this fraction.

The plasma of patients with haemophilia appears to lack the anti-haemophiliac globulin, but the electrophoretic pattern is normal in spite of its absence (Lewis *et al.*, 1946). The amounts of other components are very small. The choline esterase (Surgenor, Strong, *et al.*, 1949), makes up about 1% of the protein of fraction IV–6, which is a sub-fraction of fraction IV–4 (Table IV).

At present changes in the serum proteins characteristic of certain diseases are detected by the specific reactions of the antibody globulins that appear in these diseases. It is probable that other changes characteristic of certain types of disease will be detected by the reactions of other components, such as the reaction of the metal-binding protein with iron or the precipitation of the C-reactive protein with the C-polysaccharide. Such reactions may be less specific than those of antibodies but still be characteristic of a certain type of disease. The amounts of the fractions separated by electrophoresis or by other means will serve more as a measure of severity than as evidence of the nature of an illness. The empirical flocculation reactions, which appear to depend on quantitative as well as qualitative changes of the protein fractions, take an intermediate position, and should, in time, be replaced by less empirical tests.

The gross changes of the concentrations of albumin and γ-globulin can be detected by salting out methods. In liver disease the estimates of albumin usually agree with those found by electrophoresis. In nephrosis the concentration of albumin calculated from the electrophoretic pattern has little better claim to be called the true albumin than has the concentration obtained by salt fractionation. It remains to be seen how well the results obtained by methods proposed by Milne (1947) and Kibrick and Blonstein (1948) agree with those derived from electrophoretic patterns.

Like the E.S.R., abnormalities of the electrophoretic pattern are a measure of the clinical state of the patient rather than specific evidence of disease. The main interest of the study of abnormalities of the electrophoretic components of serum lies in the correlation of the degree of change to the clinical condition of the patient and in the investigation of the causes of these changes.

Ascending   Descending
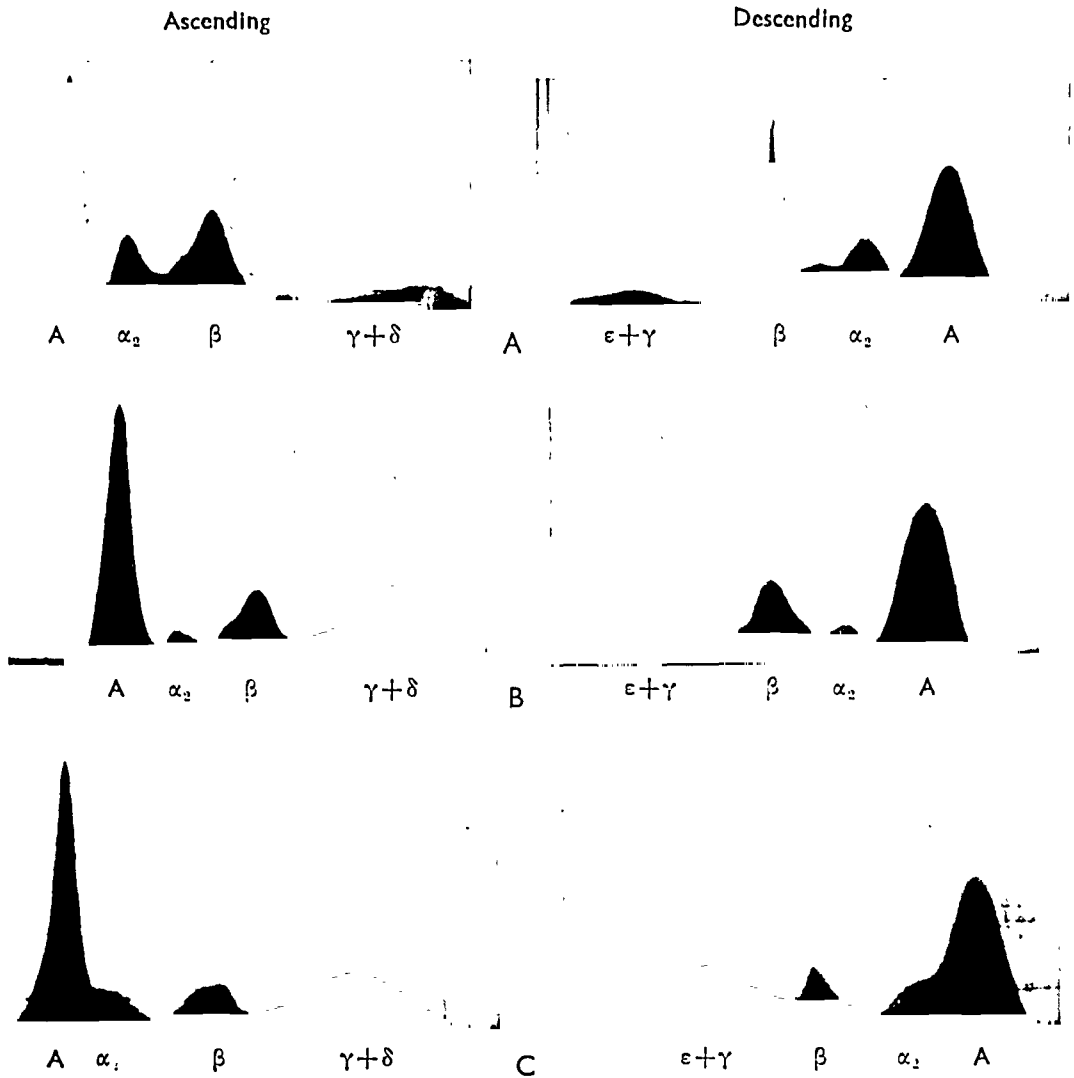
FIG. 4.—Electrophoretic patterns of (A) nephrosis, (B) cirrhosis of liver, (C) obstructive jaundice, (D1, D2) multiple myeloma, (E1, 2, 3) enlarged spleen (irradiated), (F) idiopathic hypoproteinaemia, (G) steatorrhœa, (H) kala azar, and (J) nephrosis.

ELECTROPHORETIC ANALYSES OF SERA SHOWN IN FIG. 4

	pH	Serum Diluted to g./100 ml.	Total Protein g./100 ml.	Albumin g./100 ml. (%)	Globulin g./100 ml. (%)			
					α_1	α_2	β	γ
A	8.0	1.15	4.3	1.9 (44.0)		0.7 (16.5)	1.2 (28.0)	0.5 (11.5)
B	8.0	1.25	7.3	2.9 (40.0)		0.5 (17.0)	1.4 (19.0)	2.5 (34.0)
C	8.0	1.4	7.0	3.1 (44.0)		0.7 (10.0)	1.2 (17.0)	2.0 (29.0)
D1	8.6	1.4	7.6	3.3 (43.0)	0.3 (4.0)	0.7 (9.5)	0.8 (11.0)	2.5 (33.0)
E1			5.8	2.95 (48.5)	0.6 (9.5)	0.90 (15.0)	0.95 (15.5)	0.70 (11.5)
2	} 8.6	1.4 {	6.1	2.75 (47.5)	0.5 (9.0)	0.95 (16.0)	1.00 (17.0)	0.60 (10.5)
3			6.1	2.60 (43.0)	0.75 (12.0)	1.20 (19.0)	0.95 (15.5)	0.60 (10.5)
F	8.6	1.4	4.4	2.1 (47.5)	0.4 (9.0)	0.7 (16.0)	0.9 (20.5)	0.3 (7.0)
G	8.6	1.4	5.2	1.7 (33.0)	0.7 (13.5)	1.1 (21.0)	0.7 (13.5)	1.1 (21.0)
H	8.0	0.8	8.7	2.4 (27.5)		0.4 (4.5)	0.8 (9.0)	5.1 (59.0)
J	not analysed							

A.—NEPHROSIS. History of oedema for 10 months. Blood pressure, 130/85; renal efficiency tests normal; plasma cholesterol, 0.33 g./100 ml. γ- globulin low in spite of respiratory infection one month before sample taken.
B. CIRRHOSIS OF LIVER.—Jaundice; ascites; thymol turbidity 16 units.
C. OBSTRUCTIVE JAUNDICE.—Carcinoma of pancreas; chronic hepatitis; thymol turbidity, 2.5 units; colloidal gold, 0; cephalin-cholesterol, 0.

Ascending   Descending

A α₁ α₂ β   γ δ   D I   ε γ   β α₂ α₁ A

A   γ δ   D2   ε γ   A

A α₁ α₂ β γ   δ   E I   ε γ   β α₂ α₁ A

A α₁ α₂ β γ   δ   E 2   ε γ   β α₂ α₁ A
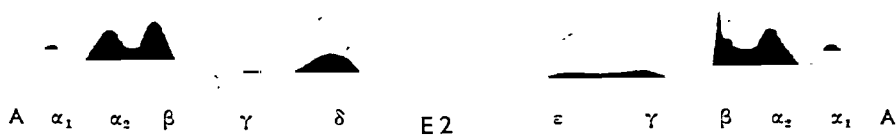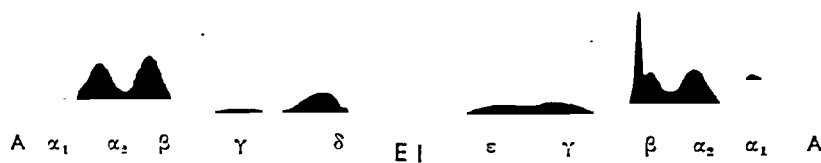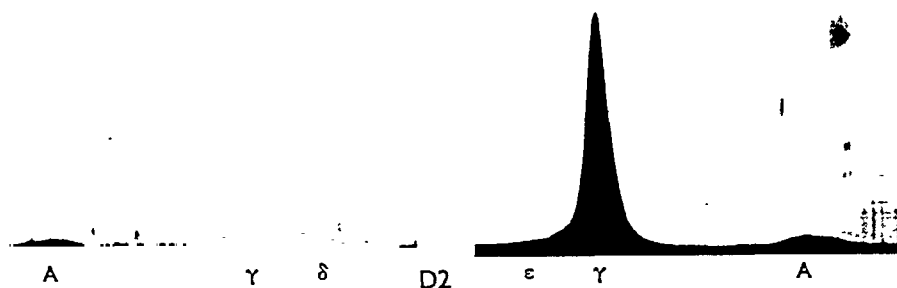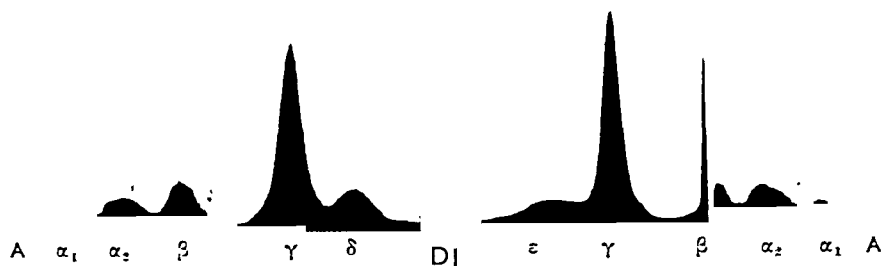
D. MULTIPLE MYELOMA (1) serum, (2) urine.—X-ray examination showed multiple punched-out areas in bones. Thymol turbidity, 3 units; E.S.R. (Wintrobe), 18 mm./1 hour. Urine contained a little albumin and much Bence-Jones protein which did not redissolve on boiling.

E. ENLARGED SPLEEN (irradiated).—(1) Before irradiation; (2) one day after irradiation (see over).

Ascending   Descending

A   α₁   α₂   β   γ   δ   E 3   ε   γ   β   α₂   α₁   A

F

G

H
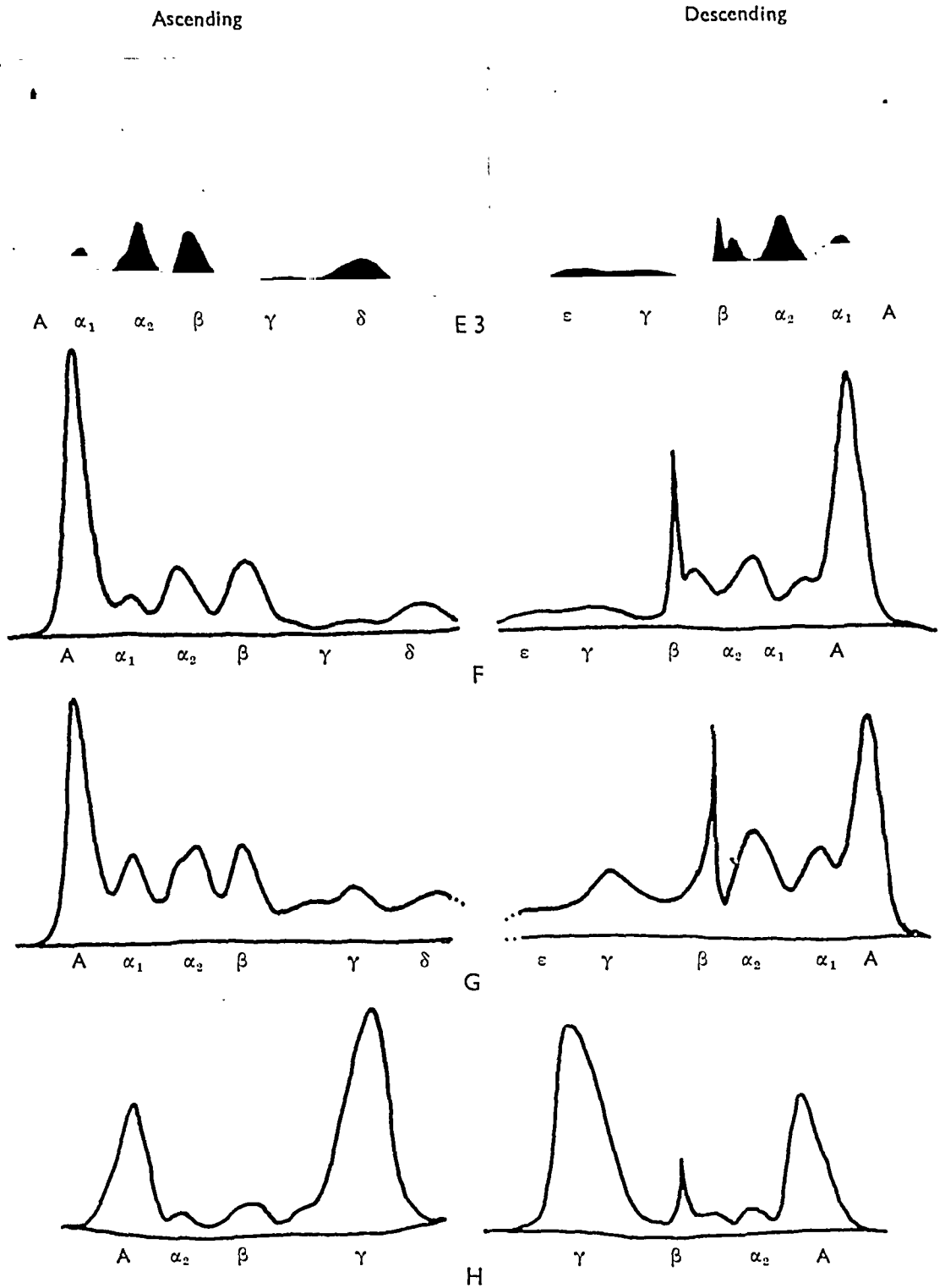
E. ENLARGED SPLEEN (irradiated).—(1) and (2) see p. 188; (3) six days after irradiation.
F. IDIOPATHIC HYPOPROTEINAEMIA (? coeliac disease).
G. STEATORRHEA.—Subject very emaciated; voluminous fatty stools; serum calcium, 6.5 mg./100 ml.
H. KALA AZAR.—Jaundice; thymol turbidity, 26 units.
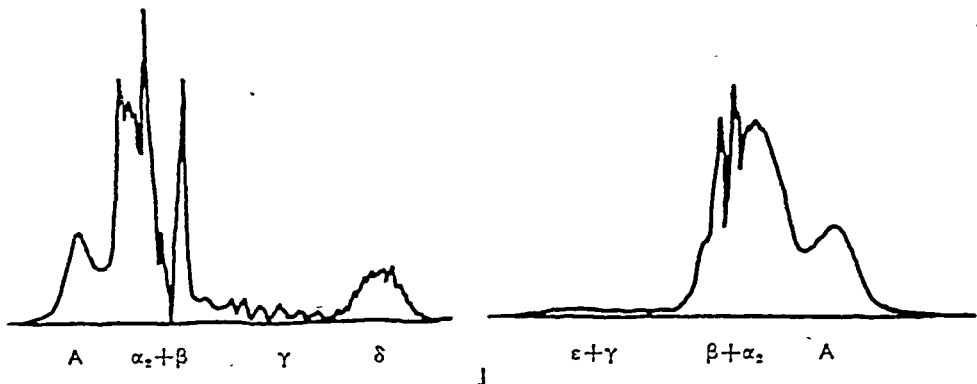
FIG. 4

J. NEPHROSIS.—Subject had oedema since pregnancy 10 months previously. Gross oedema with effusions; blood pressure, 180/100; blood urea, 42 mg./100 ml.; plasma cholesterol, 1.25 g./100 ml. Received two injections T.A.B. vaccine. Agglutinin titres, typhoid-H to 1/2560, typhoid O to 1/540, although γ-globulin low.

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SERUM COPPER LEVELS IN PREGNANCY AND IN PRE-ECLAMPSIA

BY

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In 1928 Krebs reported that the concentration of copper in the serum was significantly raised in the terminal stages of pregnancy. Since that time this observation has been confirmed by a number of workers (Gorter, Grendel, and Weyers, 1931 ; Locke, Main, and Rosbash, 1932 ; Sachs, Levine, and Fabian, 1935 ; Nielsen, 1944), who have shown that at full term the serum copper level is approximately twice the value found in non-pregnant subjects.

The significance of this raised serum copper level in pregnancy is, however, still obscure. Sachs, Levine, and Fabian (1935) have concluded that it represents a normal, physiological adjustment for the transport of copper from the maternal blood to the foetus, but no evidence is presented in support of this view. Other observations in the literature suggest that the hypercupraemia of pregnancy may, in part at least, be associated with some more specific function. It is well established that certain of the plant phenol oxidases are copper-containing proteins (Kubowitz, 1937, 1938 ; Keilin and Mann, 1938, 1939). Baker and Nelson (1943) have described a copper protein from kidney which is capable of catalysing the oxidation of adrenaline, and Holmberg in 1944 obtained a blue copper-containing protein from the " euglobulin " fraction of the serum proteins which possessed a laccase-like activity, catalysing the oxidation of *p*-phenylenediamine.

More recently, Holmberg and Laurell (1948) have described a blue protein containing 0.36% Cu derived from the serum globulin fraction which, when added to male plasma to give a final copper concentration of $700 \mu g./100$ ml., increased the histaminolytic activity of the plasma 400-fold. They also point out that the increase in serum copper in pregnancy runs parallel with the increase in the histaminolytic activity of the plasma.

In view of this finding, and since changes in the histaminolytic activity of the plasma have been reported in pre-eclamptic toxaemia (Kapeller-

Adler, 1944, 1947), a study of the level of serum copper in this condition has been carried out.

It is known from the work of Abderhalden and Möller (1928) and Boyden and Potter (1937) that the copper in the plasma from non-pregnant subjects exists in a non-dialysable form, and Eisler, Rosdahl, and Theorell (1936), using electrophoretic methods, have stated that the rate of migration of the copper in horse serum is the same as that of the serum albumin. Cohn and Koechlin (1947), on the other hand, have described a copper-combining protein in the β-globulin fraction of human plasma. Experiments have also been carried out therefore to determine the proportion of the copper precipitating with various protein fractions, separated by graded concentrations of sodium sulphate, from the serum of both non-pregnant and pregnant subjects.

Experiments

Materials.—Blood samples have been taken from 42 women with normal pregnancies (37–40 weeks), from 35 subjects four to seven weeks post-partum, from a further nine normal subjects at intervals throughout pregnancy, and from 18 healthy, non-pregnant women in the reproductive age.

Samples have also been obtained from 12 cases admitted to the maternity ward on account of pre-eclamptic toxaemia. The criteria on which this diagnosis was based were a blood pressure of over 160/90 occurring after the thirtieth week of pregnancy in a subject whose blood pressure at the beginning of pregnancy had been within the normal range, together with either albuminuria (ten cases) or oedema (nine cases).

Methods.—The samples were taken into acid-cleaned all-glass syringes, and all glassware used subsequently in the course of the estimations was acid-cleaned and washed with copper-free water distilled over " pyrex " glass.

Copper was estimated in 3 ml. of the separated serum by a modification of the colorimetric method of Eden and Green (1940), based on the reaction of copper with sodium diethyldithiocarbamate. In all experiments in which it was desired to estimate the absolute

amount of copper present, the sample was digested with H_2SO_4, HNO_3, and $HClO_4$; blank estimations were performed on identical amounts of all reagents used.

Results

The values obtained for the serum copper levels of a series of healthy, non-pregnant women, of women late in pregnancy (37–40 weeks), and of women four weeks and five to seven weeks post-partum, together with a small series from cases of pre-eclamptic-toxaemia, are shown in Table I.

TABLE I

SERUM COPPER LEVELS (μG.CU/100 ML. SERUM) IN NON-PREGNANT SUBJECTS, IN NORMAL PREGNANCY, AND IN PRE-ECLAMPSIA

Subjects	Mean	Standard Error of the Mean	Range	No. of Cases
Normal Non-pregnant ..	106	4.2	78–135	18
Normal pregnancy (37–40 weeks)	230	5.7	180–288*	42
4 weeks post-partum	143	4.5	102–180	24
5–7 weeks post-partum	114	5.4	88–139	11
Pre-eclamptic..	277†	8.1	235–337	12

* One value of 387 was obtained. Although this has been included in calculating the mean, it has not been included in the range since it differs from the mean by more than three times the standard deviation of the mean.

† Individual values in pre-eclamptics were 250, 312, 255, 337, 275, 277, 235, 281, 252, 283, 285, and 280 μg./100 ml.

It will be seen that in each case the serum copper level in the terminal stages of normal pregnancy is very considerably raised.

A further nine cases attending the ante-natal clinic were followed through pregnancy at approximately three-monthly intervals, and a further estimation was made on each when the patient returned to hospital for the final post-natal examination. Since these subjects were attending the clinics as out-patients it was not possible in each case to obtain blood samples at exactly similar intervals, and the estimations were carried out at times varying from 10 to 16 weeks, 26 to 32 weeks, and 37 to 40 weeks of pregnancy in each case, and again at 6 to 11 weeks post-natally. The results of these estimations are given in Table II.

The findings of these nine cases confirm the earlier observations of Nielsen (1944) that the rise in serum copper occurs at an early stage in pregnancy; it will be seen that the level is significantly increased by the tenth to sixteenth week, although a further slight increase appears to take place until full term; 6 to 11 weeks after delivery the serum copper level appears to have returned once more to the normal level for the non-pregnant subject.

TABLE II

SERUM COPPER LEVELS (μG.CU/100 ML. SERUM) THROUGH-OUT NORMAL PREGNANCY AND POST-NATALLY

Case	10–16 Weeks	26–32 Weeks	37–40 Weeks	Post-natal (6–11 Weeks)
1	267	298	288	185
2	182	—	287	129
3	193	270	228	89
4	152	178	(387)*	133
5	182	228	212	126
6	148	190	240	117
7	166	213	237	95
8	183	225	240	110
9	179	165	215	92
Mean	184	221	243	119

* Not included in the mean given in this table since it differs from the mean given in Table I by more than three times the standard deviation of the mean.

The values obtained in pre-eclampsia are also high, the mean for the 12 cases studied being 277 μg./100 ml. (standard error = 8.1) compared with a mean of 230 μg./100 ml. (s.e. = 5.7) for normal full-term pregnancies. Moreover, the standard error of the difference between these means is 11.5 giving t = 4.1 for n = 52. It should be pointed out that in calculating the standard error of the normal pregnant group the value of 387 μg./100 ml. has been included in the series, even though this differed from the mean by more than three times the standard deviation of the mean.

From the results which we have obtained on this small series of cases it would appear that in pre-eclampsia the serum copper level shows a small but statistically significant increase over the normal elevation occurring in pregnancy.

In order to obtain information as to the physiological significance of this extra serum copper in pregnancy it was decided to carry out some initial experiments to determine, by means of fractional precipitation of the serum proteins, whether this excess copper in the serum behaves in a similar fashion to the copper present in the serum of non-pregnant subjects, and also to obtain evidence as to which of the main groups of protein the copper is combined.

Total globulins were first precipitated with 26.8% Na_2SO_4 (Majoor, 1947; Milne, 1947), and the percentage of the total serum copper precipitating with the globulins determined. In these fractionation experiments advantage was taken of the finding by Tompsett (1934) that the serum copper can be quantitatively estimated on trichloroacetic acid filtrates of whole serum, acidification releasing the copper from its bound form.

The globulins, precipitated from 3 ml. of serum by means of 26.8% Na_2SO_4 (previously washed with ammoniacal sodium diethyldithiocarbamate, extracted with amyl alcohol, recrystallized and dried in the oven at 110° C.), were therefore dissolved in H_2O and re-precipitated with a final concentration of 5% trichloroacetic acid; after standing for 10 minutes the precipitate was centrifuged, and the supernatant transferred to a separating cylinder for copper estimation. The precipitate was washed once with 3 ml. 5% trichloroacetic acid, and the supernatant also added to the separating cylinder. Total serum copper was estimated on the filtrate obtained by precipitation of 3 ml. serum with trichloroacetic acid in the same manner.

In addition, Milne (1947) has shown that 19.6% Na_2SO_4 yields a precipitate of " euglobulin " which appears to correspond reasonably quantitatively with electrophoretically estimated β- + γ-globulins, and Kibrick and Blonstein (1948) have claimed that a fraction agreeing satisfactorily with electrophoretically estimated γ-globulin can be obtained by precipitation with 15% Na_2SO_4. An attempt was made therefore to estimate the copper in fractions precipitated with 15%, 19.6%, and 26.8% Na_2SO_4 in the hope of being able thereby to determine roughly the percentage of the total serum copper bound with the α-, β-, and γ-globulin fractions respectively. Some difficulty was experienced, however, in obtaining satisfactory flocculation for either filtration or centrifugation of the precipitates obtained at these salt concentrations without the use of high dilutions of the serum which would have introduced inaccuracies into the subsequent copper estimations. For this reason ether was added in order to obtain a compact and centrifugable separation of the precipitated globulins (Kingsley, 1940).

The procedure finally adopted was as follows: Serum, 3 ml., is added slowly to the weighed quantity of solid copper-free Na_2SO_4 contained in a round-bottomed centrifuge-tube which is stirred mechanically and immersed in a water-bath at 35° C. After stirring for five minutes the tube is removed from the bath, 1 ml. of ether added, and the tube shaken for 20 to 30 seconds. It is then immediately centrifuged for 10 minutes at 3,000 revolutions per minute. The ether is then poured off, and the lower aqueous layer removed with a teat-ended capillary pipette and discarded. The precipitate is dissolved in 4 ml. of water and re-precipitated with trichloroacetic acid as described above.

The percentages of the total serum copper precipitating with the various globulin fractions obtained by these means are shown in Table III. Using only Na_2SO_4 as the precipitating agent it will be seen that 89% of the copper present in the serum from non-pregnant subjects appears in the precipitate of total globulins; when Na_2SO_4 together with ether was used, the globulin precipitate contained 86% of the total copper present in the serum, a value not differing significantly from that obtained with Na_2SO_4 alone. A few experiments were also done to compare the effects of the use of ether on the fractions precipitating with 15% and 19.6% Na_2SO_4, but again it was found that the presence of ether did not affect the amount of copper precipitating at these concentrations. Although no electrophoretic study of these protein fractions has been made, it has therefore been concluded that we may justifiably consider these fractions as representing roughly the α-, β-, and γ-globulin fractions described by Kibrick and Blonstein (1948).

TABLE III

PERCENTAGES OF TOTAL SERUM COPPER PRECIPITATING WITH VARYING GLOBULIN FRACTIONS

Subjects	Precipitant			
	Na_2SO_4(%) + Ether			Na_2SO_4 (%)Alone
	15	19.6	26.8	26.8
Normal, non-pregnant	33	82	75	94
	21	79	97	95
	38	74	85	86
	16	81	86	86
	23	68	89	86
Mean % of copper ..	26	77	86	89
Pregnant (37–40 weeks)	31	84	98	
	29	81	94	
	22	80	100	
	22	76	85	
	25	81	100	
	24	—	86	
	22	—	90	
Mean % of copper ..	25	80	93	

The results summarized in Table III show that no significant difference exists between non-pregnant and pregnant sera as regards the amount of copper appearing in the protein fractions precipitated by 15%, 19.6%, and 26.8% Na_2SO_4. Further, using Kibrick and Blonstein's interpretation of these protein fractions, it appears that approximately 25% of the copper is combined with the γ-globulin fraction, 55% with the β-globulins, and a further 10% with the α-globulins.

Discussion

The considerable increase in the serum copper level in pregnancy observed by earlier workers has been confirmed. The main object of the present work was to discover whether this elevated serum copper level undergoes any change if a pre-eclamptic toxaemia develops in the course of the pregnancy. Although the number of cases of pre-eclamptic toxaemia which we have been able to study is small, the results show that in this condition there appears to be a small but statistically significant increase in the serum copper level over

the normal elevation which occurs in a healthy pregnancy. From the few cases we have studied there does not appear to be any obvious evidence of a correlation between the severity of the toxaemia and the height of the serum copper level, although further work on a larger series of cases would be needed to establish this point. It is clear, however, that the decrease in histaminolytic activity of the plasma in pre-eclamptic toxaemias, which has been reported by Kapeller-Adler (1944, 1947), is not accompanied by any corresponding fall in the level of serum copper.

With a view to obtaining information about the state and physiological significance of the raised serum copper in pregnancy, information has been obtained as to relative amounts of copper bound to the various fractions of the plasma proteins. Our finding that 90% of the serum copper appears in the globulin precipitate produced by 26.8% Na_2SO_4 agrees well with the proportion precipitated by 50% saturation with $(NH_4)_2SO_4$ (Holmberg and Laurell, 1947). By using graded concentrations of Na_2SO_4 it has been shown that the greater part of this globulin-bound copper exists in combination with those globulins precipitating between 15% and 19.6% Na_2SO_4, that is with a fraction which has been claimed by Kibrick and Blonstein (1948) to consist largely of β-globulins. In this connexion it is of interest that Longsworth, Curtis, and Pembroke (1945) have shown by electrophoretic analysis that in pregnancy there is a decrease in the serum albumin level accompanied by an approximately two-fold increase in the β-globulin level. Novak and Lustig (1947) have also reported a significant fall in the albumin/globulin ratio of the serum in pregnancy, and have claimed that this ratio is still further reduced in toxaemic patients, a finding which accords with our demonstration of a further rise in the serum copper level in pre-eclamptic toxaemias. Moreover, a β-globulin has recently been isolated from human plasma which is capable of combining with iron, copper, or zinc, and has been designated the " metal-combining globulin " (Cohn and Koechlin, 1947 ; Surgenor, Koechlin, and Strong, 1949). The properties of this metal-combining protein have so far mainly been studied in connexion with its combination with iron. Although the present findings are not concerned specifically with this protein, they indicate that not only does the larger part of the copper normally present in human serum also exist in combination with a β-globulin, but that the extra copper present during pregnancy appears to show the same distribution among the major fractions of the plasma proteins. Our results with human serum are therefore in contrast with the findings of Eisler, Rosdahl, and Theorell (1936) that the copper in horse serum is largely associated with the albumin fraction.

Summary

Estimations of the serum copper level have been carried out in blood samples obtained from healthy, non-pregnant women, from women late in pregnancy, from women four to eleven weeks post-partum, and in 12 cases of pre-eclamptic toxaemia.

The results indicate that in pre-eclampsia the serum copper level shows a small but significant increase over the normal elevation occurring in pregnancy.

Approximately 90% of the serum copper appears in the globulin precipitate produced by 26.8% Na_2SO_4.

By the use of graded concentrations of Na_2SO_4 the copper partition among various globulin fractions has been determined.

No significant difference was observed between samples from pregnant and non-pregnant subjects in the distribution of the protein-bound copper.

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LATENT CARCINOMA OF THE PROSTATE

BY

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The incidence of carcinoma of the prostate in 1947 in the United Kingdom, according to the Registrar-General, was 0.98% of all male deaths. Nevertheless several writers (Muir, 1934; Moore, 1935; Rich, 1935; Gaynor, 1938; Kahler, 1939; Baron and Angrist, 1941; Luppi, 1947) have reported a much higher incidence of carcinoma in prostates removed at routine necropsy when the condition was unsuspected. The reported figures range from 2.1% to 46%. This variation can only be attributed to differences of method of examination or of criteria of malignancy. Since it is important to establish the true incidence of latent carcinoma of the prostate it was decided to make a thorough study of prostates removed at necropsy, and to employ a careful and uniform technique.

Material and Methods

One hundred and forty-two prostates were studied. These had been removed at necropsy from males between the ages of 15 and 79 years in whom the diagnosis of carcinoma of the prostate had not been made clinically or on gross examination at necropsy.

Each prostate was dissected free from surrounding tissue and its volume measured by reading the volume of fixing fluid displaced by the gland on immersion in a measuring cylinder. It was then fixed whole for ten days in 10% formol saline (one part commercial formaldehyde with nine parts normal saline), buffered to pH 7.0, the solution being changed on the second day. Fixation with corrosive formol was tried at first, but formol saline gave better nuclear detail and was satisfactory even with the largest gland with a volume of 105 ml. Prostates were obtained up to 54 hours after death.

The glands were first cut coronally through the urethra, thus dividing them into anterior and posterior portions. Each portion was then placed on the machine used for slicing the brain. This consists of the base and three sides of a shallow wooden box with sheets of glass within it. The number of glass sheets was adjusted to allow a space of 4 mm. between the top of the box and the surface of the glass, so that a long knife laid on the top of the box could be used to cut slices 4 mm. thick. In this manner the prostate was completely divided into blocks about 4 mm. thick, each of which was numbered. Since the

prostates varied considerably in size and shape it was sometimes more convenient to section them sagittally, but in every case the whole gland was divided into blocks. Some variation in the thickness of the blocks was inevitable, but none was thicker than 5 mm. Blocks thinner than 4 mm. were difficult to work with, particularly if they were large, as they became distorted.

Dehydration and clearing were carried out slowly with an alcohol-butyl-benzene series. The blocks were embedded in paraffin and sections cut at a thickness of 8 μ from the same surface of each block. When small calculi were encountered during cutting use was made of Ebner's solution as advised by Lendrum (1947). As a routine measure the sections were stained with Weigert's haematoxylin and 1% alcoholic eosin.

By this method a series of sections was obtained from each prostate, the sections being at roughly 4 mm. intervals right through the gland. Moore (1935) calls this a method of "step-section."

Criteria of Malignancy

Since adenocarcinoma of the prostate is often composed of small, well-differentiated acini in which nuclear changes and mitotic figures are rare or absent, it is necessary to scrutinize carefully the relationship of epithelium to stroma. Prostatic acini whether normal or hypertrophic are surrounded by a stroma propria, composed of fibromuscular and a little elastic tissue, which closely invests them and faithfully follows the contour of the acinus even into the projecting papillae. Together the acinus and the stroma propria form a single unit around which the supporting stroma of the gland sweeps (Fig. 1). Most normal and hypertrophic acini also possess a basal layer of epithelial cells which are flattened or fusiform in shape and are arranged horizontally around the acinus. There are, however, certain acini which do not possess a basal layer of cells, but these still retain the normal relationship to the stroma propria (Fig. 2). Malignant acini have neither a basal layer of epithelial cells nor a stroma propria. The absence of a basal layer, therefore, does not necessarily indicate malignancy, but the relation-

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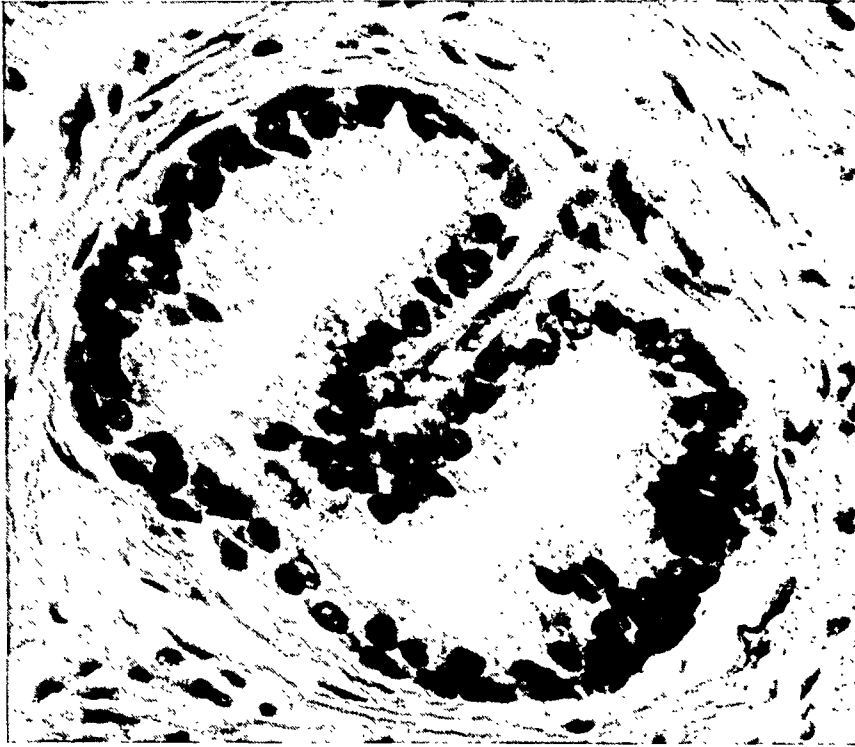
FIG. 1.—Normal prostatic acinus with basal layer of epithelial cells and stroma propria which can be followed into the papilla. × 550.

invasion, and invasion of the blood vessels. Perineural lymphatic invasion was looked for particularly in every case.

Results

Carcinoma was found in 17 (12%) of the 142 prostates examined. It was not found before the age of 40. The incidence after 40 is shown in Table I. Statistical analysis shows that there is a significant rise in the incidence of carcinoma between the sixth and seventh decades ($P < 0.01$) but not beyond the seventh. This does not agree with the observation of Moore (1935), who concluded that the incidence of carcinoma increased steadily up to a maximum at the ninth decade ; critical examination of his figures shows that the increase was not really statistically significant.

ship of the acinus to its stroma is of great importance and is particularly striking when malignant acini are invading muscle.

Moore (1935) drew attention to the tendency of carcinoma of the prostate to spread by the perineural lymphatics, and Kahler (1939) describes this as the most valuable criterion in its diagnosis. This form of infiltration is seen frequently and extensively even in small tumours.

In view of the special characteristics of carcinoma of the prostate the diagnosis of malignancy was based on the observation of abnormal stromal relationship together with any three of the following: nuclear changes, mitotic figures, anaplasia, heteroplasia, lymphatic

FIG. 2.—Normal acinus without basal layer of epithelial cells but with normal stroma propria. × 550.

TABLE I

INCIDENCE OF CARCINOMA AND BENIGN HYPERTROPHY

Age Groups	Total Number of Prostates	Prostates without Carcinoma	Prostates with Carcinoma	Prostates with Benign Hypertrophy
40–49 ...	22	21	1	2
50–59 ...	38	36	2	23
60–69 ...	39	32	7	37
70–79 ...	22	15	7	22

Site of Origin.—Lowsley (1930) has shown that the prostate begins to develop during the third month of foetal life as five groups of solid outgrowths from distinctive parts of the urethra. One group arises from the posterior wall of the urethra between the bladder neck and the ejaculatory ducts : one on each side from the lateral furrows of the urethra including the lower part ; one from the posterior wall of the urethra below the ejaculatory ducts ; and one from the upper and anterior part of the urethra. A middle lobe, two lateral lobes, a posterior lobe, and an anterior lobe develop from these outgrowths. The middle lobe is bounded by the bladder superiorly, the urethra anteriorly, and the ejaculatory ducts posteriorly ; its ducts open into the posterior wall of the urethra above the ejaculatory ducts. The lateral lobes are situated on each side of the urethra and extend posteriorly and anteriorly, and their ducts pass posteriorly, then medially and anteriorly to end in the lateral furrows of the urethra. The posterior lobe is bounded anteriorly by the urethra, anterosuperiorly by the ejaculatory ducts, and posteriorly by the capsule ; its ducts end in the posterior wall of the urethra below the ejaculatory ducts. The anterior lobe is small and is situated in the median plane anterior to the upper part of the urethra ; its ducts end in the anterior wall of the urethra. The middle lobe may be absent occasionally. Lowsley states that the acini of the middle and lateral lobes may intermingle, but considers that a distinct layer of fibrous tissue separates the posterior and lateral lobes. Whether or not it is accurate to regard the prostate as sharply divided into lobes is debatable, but the divisions do serve as a rough means of siting lesions.

In one prostate the tumour was so extensive that it was impossible to determine its site of origin, and this was the only gland in which the middle lobe was involved and the seminal vesicles infiltrated. In four glands two separate areas of carcinoma were found. In two of these there was considerable difference in the size of the tumours, but in the other two the tumours were of similar size, thus supporting the view that prostatic carcinoma may be multifocal in origin (Moore, 1935). It was possible, therefore, to localize with fair accuracy the sites of origin of 20 small carcinomata. Twelve were confined to, or occurred maximally in, the posterior lobe and eight arose in the lateral lobes. This confirms the finding that carcinoma of the prostate occurs most commonly in the posterior lobe. The anterior lobe did not contain tumour in any case.

Method of Spread.—Eleven tumours in the posterior lobe and two in the lateral lobes arose just beneath the capsule. and in eight of these the capsule was infiltrated, thus supporting the observations of Rich (1935) and Gaynor (1938).

In all but four glands the tumours were confined to one lobe : of these four, two, arising in the posterior lobe, had spread forwards on both sides of the urethra into the anterior part of the gland, but the other two, arising in the lateral lobes, had spread forward around the urethra into the lateral lobe on the other side.

Size of Tumours.—All the tumours except one could be roughly measured. The largest was $20 \times 20 \times 8$ mm. and the smallest $2 \times 2 \times 2$ mm. The tumours varied considerably within this range, the average volume being 718 c.mm.

Histology.—The tumours were all adenocarcinomata which varied in histological appearance but had certain features in common. In the main they were composed of small acini. Since Luppi (1947) has stated that senile acini and acini distorted by the pressure of " adenomata " in benign hypertrophy may be wrongly diagnosed as malignant, it is important to emphasize the differences between malignant and non-malignant small acini.

Small senile acini are shrunken and often distorted. They are usually lined by small irregular cubical or flattened cells with small pyknotic round or irregular nuclei (Fig. 3a). Cells with thin oval nuclei usually arranged perpendicularly to the lumen also occur and may predominate (Fig. 3b). The basal layer of cells usually has rounder and paler staining nuclei. In older glands the luminal cells are all flattened and the basal nuclei narrow and pyknotic similar to those of the lining layer (Fig. 3c). The cell outline is indefinite and the cytoplasm scanty, palely eosinophilic. and finely granular. sometimes being reduced to a thin rim around the nucleus. The stroma propria is always present and is thickened and collagenous.
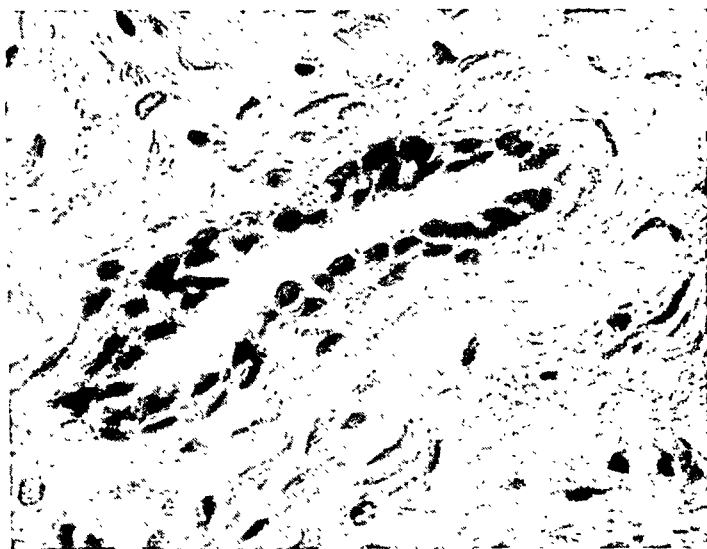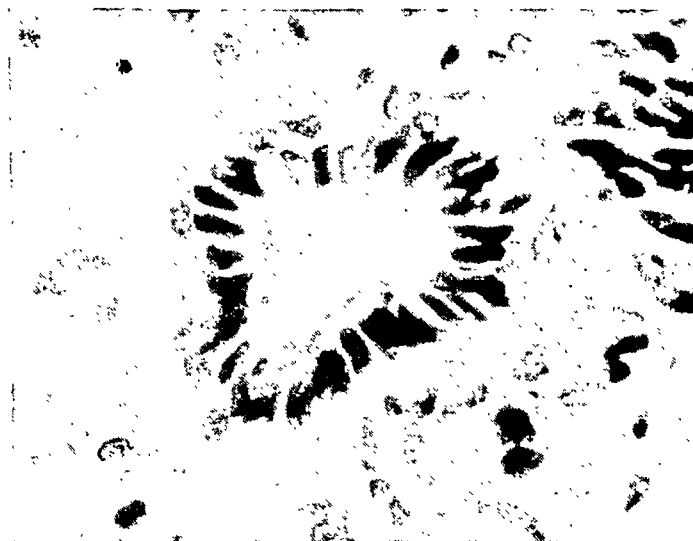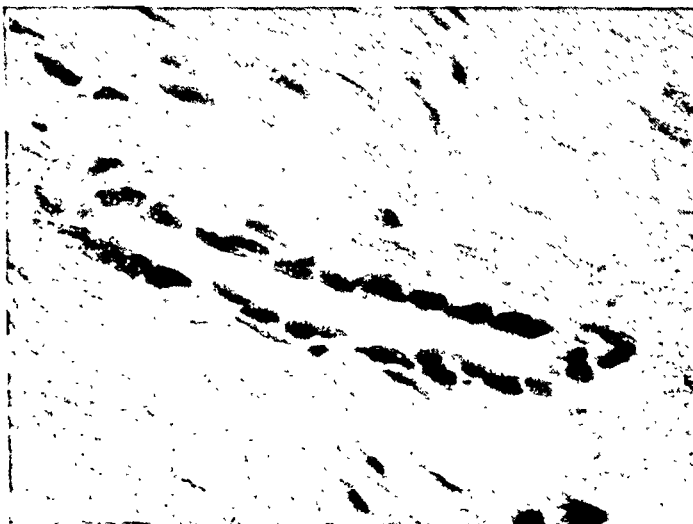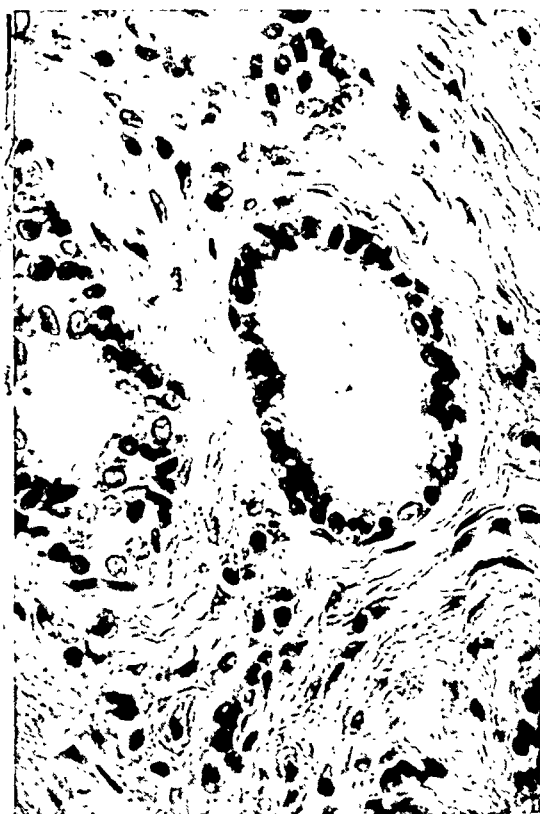
FIGS. 3a, 3b, and 3c.—Small senile acini showing small pyknotic nuclei. × 800. 3d and 3e.—Small acini in benign hypertrophy showing typical nuclei and cellular appearance with perinuclear vacuolation. d × 400, e × 800.

Prostates with benign hypertrophy contain small senile acini, but other small acini are also present which are round or oval in shape, often in contact with one another and arranged in groups. These acini are lined by cubical cells with a finely granular, deeply eosinophilic cytoplasm or low columnar cells with clear reticular cytoplasm often with a faint eosinophilic rim at the luminal border. The cells are regular and the cell borders clear cut except when secretion is occurring. The nuclei are large, round or oval, and pale staining, with a delicate chromatin network. Perinuclear vacuolation of the cytoplasm often occurs (Fig. 3d). The basal cells possess thin oval, pyknotic nuclei which form a definite rim to the acinus (Fig. 3e). The stroma propria is wide and rather cellular, and sometimes there is a thin rim of hyaline material around the acinus between the basal layer and the stroma propria.

Small, distorted acini which are seen around the "adenomata" in benign hypertrophy are unusual only in shape: their histological picture does not differ from that of the acini described above.

The small malignant acini which formed the main part of the tumours in this series were usually composed of cubical cells, but they varied in size and shape, some being rather flattened, but in one tumour giant forms were present. The malignant cells were definitely larger than those of the senile acini and were comparable in size to those of the small acini in benign hypertrophy but differed from these in their irregular shape and irregular luminal border. The cytoplasm varied: it was usually finely granular and eosinophilic but not so deeply staining as that of the small acini in benign hypertrophy. A small amount of eosinophilic secretion was occasionally present in the lumen of the acini. It is perhaps noteworthy that corpora amylacea were never seen in the carcinomatous areas. The nuclei of the malignant cells were larger than those seen in normal or hypertrophied

prostates and were larger in relationship to the size of the cell. They were usually round but occasionally oval and varied in staining power. They had a definite chromatin network which was coarser than that of the nuclei in benign hypertrophy, and there was some condensation of the chromatin at the periphery of the nucleus. One or two prominent nucleoli were usually present. The basal layer of epithelial cells was never present, and the altered relationship to the stroma was most striking. The stroma propria was absent and the malignant acini had grown into the supporting stroma separating the muscle fibres and in many cases actually rupturing them (Fig. 4). These heteroplastic acini were present in every tumour, but, in addition, other small acini occurred which had the clear reticular cytoplasm of the normal adult epithelium, and gradation could be seen between these and the more usual eosinophilic cytoplasm, but even in these the large typical nucleus was usually present and the basal layer and stroma propria were always absent.
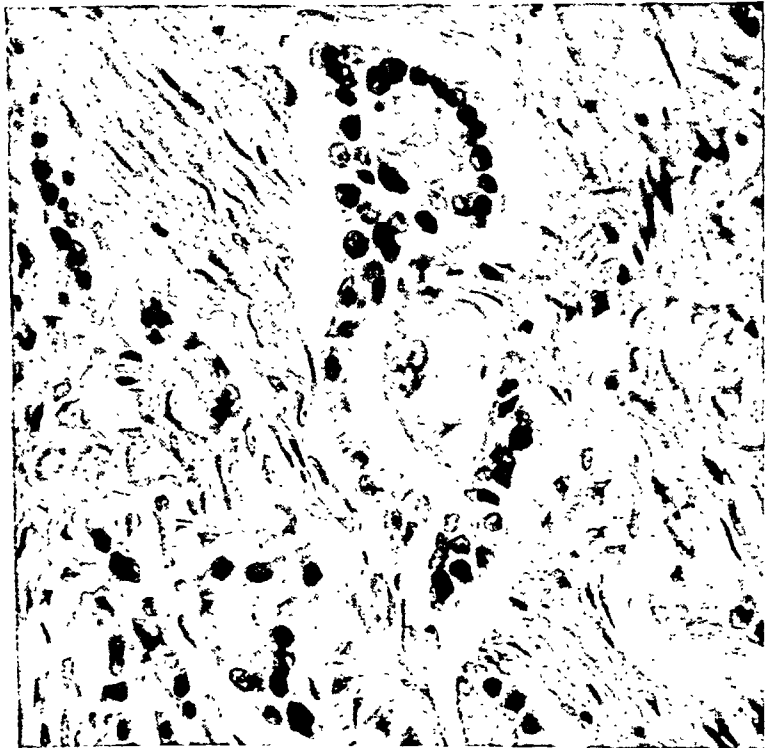
FIG. 4.—Typical small malignant acini rupturing a muscle bundle. × 400.
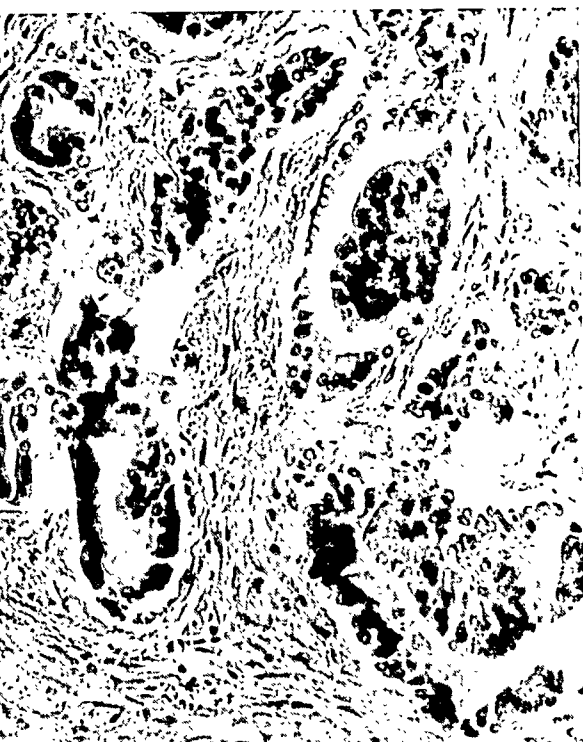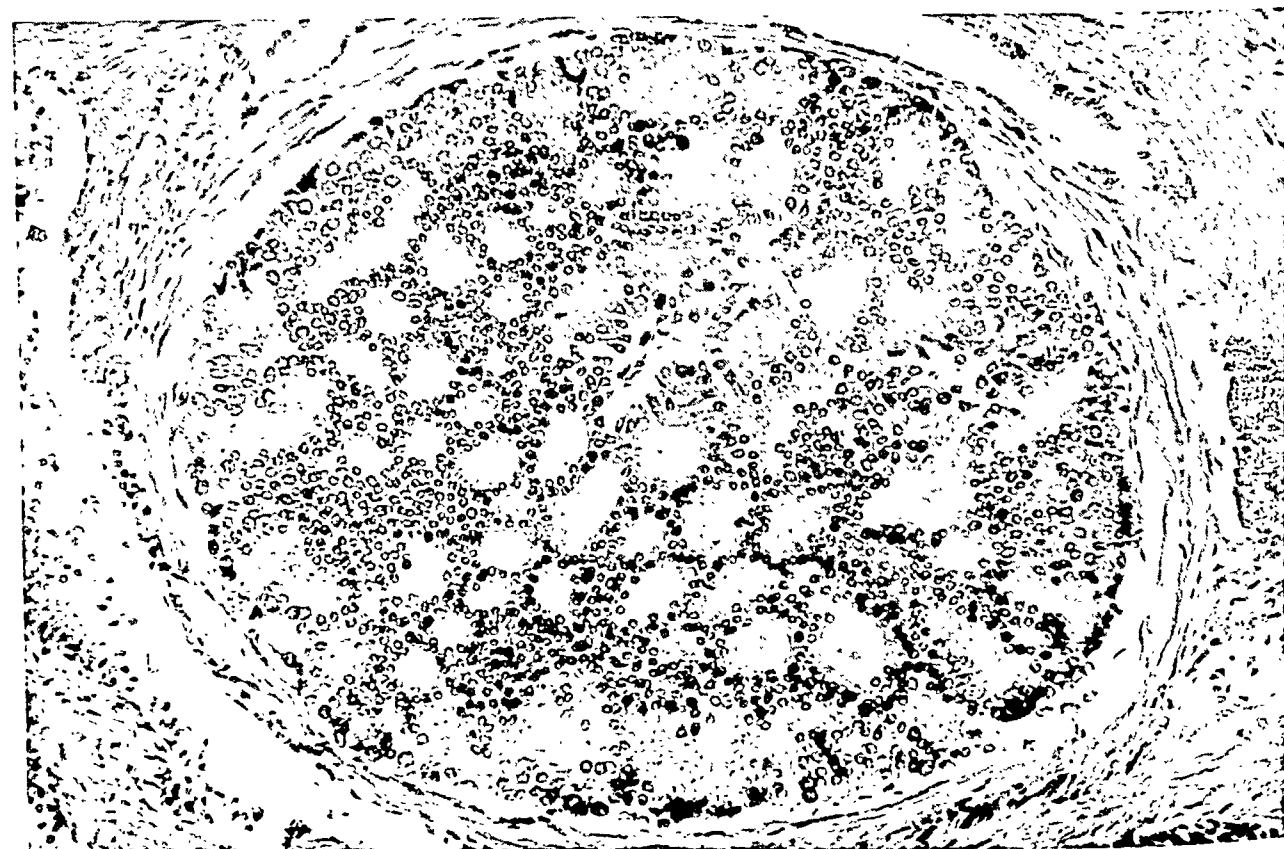
FIGS. 5a, 5b, 5c.—Intra-acinar type of carcinoma. Note appearance of false capsule around c. a × 260. b and c × 200.

The malignant acini were usually round or oval in shape, but many were distorted, the lumen having been reduced to a slit or absent, and the acinus consisted of two parallel rows of cells. This appearance depended, in some tumours, on the degree of de-differentiation and was commonly associated with areas of anaplasia. The cells lining these acini were large and irregular and appeared to be thrusting out processes into the surrounding stroma. Small aberrant acini also occurred composed of two crescentic cells joined at either pole with a lumen between them. In other tumours the distorted appearance was the result of stromal reaction produced by the tumour. A marked scirrhous reaction was present in four of the tumours

There were anaplastic areas in 14 of the tumours. These areas were in the main small, but in four they formed a considerable bulk of the tumours. In one tumour the cells had undergone mucoid degeneration and presented the typical signet ring appearance.

In addition to the small acini six tumours contained larger acini which showed intra-acinar proliferation of cells with a tendency to form small acini within the main acinus. The degree of proliferation varied. Some of the acini were small with one part of the wall composed of many layers of cells, or one or more cellular papillae projecting into the lumen (Fig. 5a). Others were much larger and were composed of large masses of cells with many small lumina within them so that they appeared as aggregations of small acini (see Fig. 5c) and resembled cribriform carcinoma of the breast (Muir, 1941). Every gradation could be seen between these two types, the common form being a large acinus with multiple cellular papillae, without a stromal core, often branched and united with their neighbours (Fig. 5b). None of these acini had a basal layer or a stroma propria, but the largest, apparently growing mainly by expansion, had compressed the surrounding stroma and produced a false capsule around the acinus. The cells forming these larger acini were similar to those forming the smaller acini in the same tumour, but they were usually slightly larger and more regular, the cytoplasm usually being eosinophilic and slightly granular, but again gradation could be seen between this and the clear reticular cytoplasm of adult prostatic epithelium. All the cells in the acinus had the same cytoplasmic appearance ; there was no differentiation of luminal cells. The nuclei were similar to those of the small acini. Eosinophilic secretion was sometimes present within the lumen.

Abnormal nuclear forms occurred both in the small and the large malignant acini. These were mainly large and intensely pyknotic, but degenerate shrunken pyknotic nuclei with a crenated border were also present. These nuclear changes occurred in 16 of the 17 prostates with carcinoma. Mitotic figures were not seen in any tumour.

Perineural lymphatic invasion was present in 15 of the 17 prostates with carcinoma, and in five it was extensive. Most commonly there were one or two small heteroplastic acini crescentic in shape closely applied to the nerve (Fig. 6a). Sometimes longitudinal section of a nerve showed many small acini occupying the lymphatics along the whole
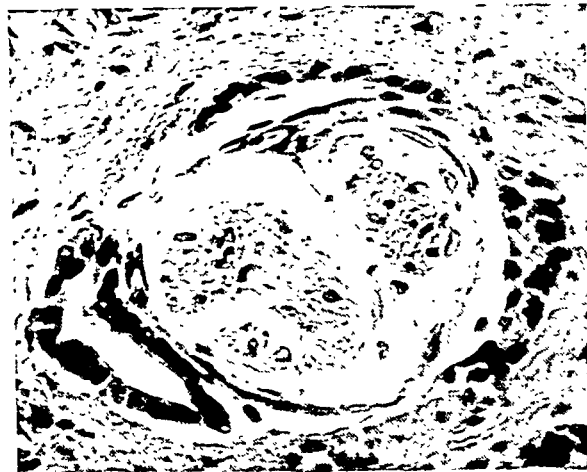
FIG. 6a.—Common type of perineural lymphatic invasion. × 200.

length of the nerve. It was often remarkable that the perineural lymphatics were invaded by the most differentiated acini in the tumour. Occasionally the lymphatics were filled with the intra-acinar type of growth (Fig. 6b), showing that, although this type of growth appears to be mainly intra-acinar, the acini are definitely invasive. The extent of perineural lymphatic invasion appeared to depend more on the size of tumour than on the degree of anaplasia ; in the most anaplastic tumour it was only found after a prolonged search. It could be found, however, in the smallest tumours and must be considered an important and early method of spread of carcinoma of the prostate.

Invasion of blood vessels was not common. The malignant acini were often seen growing in the loose connective tissue around the blood vessels, but in only four glands was there definite evidence of invasion of the wall of small vessels, and in only one of these were tumour cells actually seen in the lumen. In one other gland the wall of a large vein had been invaded.

FIG. 6*b*.—Intra-acinar type of carcinoma in perineural lymphatics. × 200.

Associated Pathological Conditions.—No pathological condition outside the prostate was found to have a significant connexion with carcinoma of that organ, but it is interesting that three of the patients with carcinoma of the prostate had carcinoma in other organs: these were carcinoma of the head of the pancreas with metastases, carcinoma of the colon with metastases, and a small carcinoma of the rectum. None of these tumours bore any resemblance histologically to the incidental carcinoma of the prostate and therefore could not be confused with it. The incidence of carcinoma in other organs in the whole series of 142 was 47. (In 500 consecutive routine necropsies on males over the age of 15, performed before and during this investigation, the incidence of carcinoma, excluding carcinoma of the prostate, was 134.)

Calculi were present in 31 of the 142 prostates studied, three of which also contained carcinoma, but in no case did the carcinoma actually arise in an area containing calculi. Similarly 22 of the 142 prostates showed duct obstruction, focal atrophy, and acute inflammation; three of these also contained carcinoma, but in only one did the carcinoma actually arise near an area of inflammation. There is therefore no significant connexion between carcinoma and prostatic calculi or inflammation.

Sixteen of the prostates with carcinoma also showed the changes of benign hypertrophy. The seventeenth, from a man aged 49, although large (volume, 30 ml.) did not show the histological changes of benign hypertrophy. Benign hypertrophy was not found in any gland before the age of 40, and its incidence after that age is compared with carcinoma in Table I. Statistical analysis (Table II) shows that benign hypertrophy and

TABLE II

INCIDENCE OF NORMAL AND CARCINOMATOUS PROSTATES WITH/WITHOUT BENIGN HYPERTROPHY OVER AGE OF 40

Condition	Prostates with Carcinoma	Prostates without Carcinoma	Total
Prostates with benign hypertrophy ...	16	68	84
Prostates without benign hypertrophy...	1	36	37
Total	17	104	121

$0.05 > P > 0.01$

carcinoma occur together more commonly than can be explained by chance. There is a significant connexion between the two conditions; the coefficient of contingency calculated from this table is 0.187 and the coefficient of correlation is 0.207; these values give some indication of the degree of dependence of the two conditions.
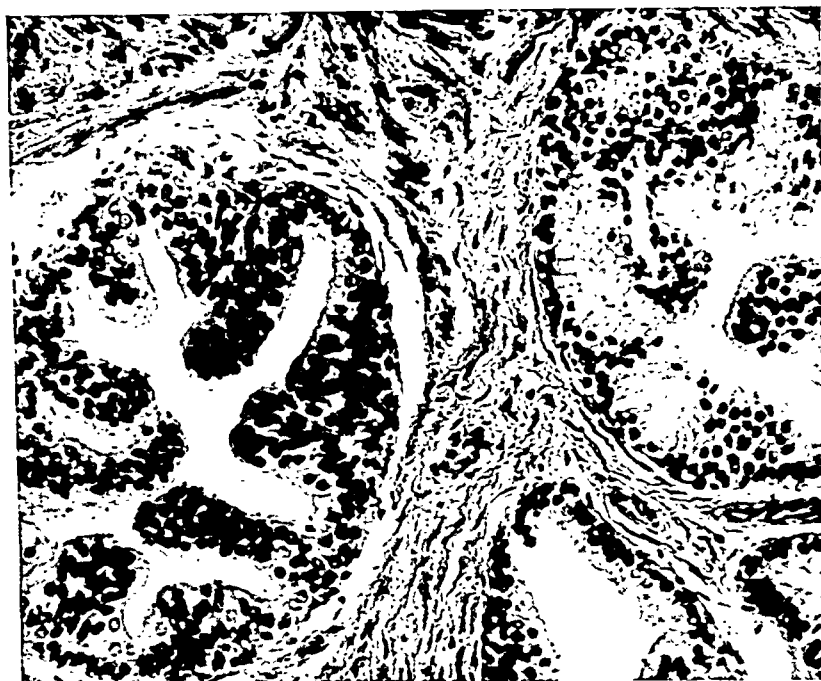
FIG. 7.—Hyperplastic acinus on left compared with normal acinus on right. × 260.

Table III shows the mean volumes of normal, hypertrophied, and carcinomatous glands. The volumes of some prostates examined were not recorded, as they had remained in fixative for a long time and shrinkage might have occurred.

TABLE III

MEAN VOLUMES AND THEIR STANDARD DEVIATIONS OF NORMAL AND ABNORMAL PROSTATES

Condition	Number of Prostates	Mean Volume (ml.)	Standard Deviation
Normal	49	21.4	6.389
Benign hypertrophy without carcinoma	63	30.35	9.277
Carcinoma	16	41.56	24.67
Total	128		

The small number of glands with carcinoma prevents accurate analysis of this table, but it is interesting that carcinoma occurred more commonly in the larger glands. Except in one case the presence of the carcinoma made very little difference to the volume of the gland, at the most

2–3 ml. It would appear that the degree of benign hypertrophy was greater in glands with carcinoma than in those without it.

Precancerous Conditions.—In 12 prostates with carcinoma a characteristic type of hyperplasia was intimately associated with the tumours. The hyperplastic acini were large and composed of large cells, columnar in form, with deeply eosinophilic cytoplasm, the nuclei being strikingly large and usually oval in shape, although occasionally round, with a well-defined though delicate chromatin network, and situated centrally in the cell. The nuclei were sometimes elongated, filiform in shape and more pyknotic. Mitotic figures were sometimes seen. The acini were lined by more than one layer of cells, and many large cellular papillae projected into the lumen. The cell outlines were blurred except that the luminal borders and the papillae appeared as solid eosinophil projections with nuclei scattered within them. The papillae were nearly always cellular ; very rarely did they have a stromal core. These acini usually had a basal layer of epithelial cells and always had a normal stroma propria (Fig. 7). Occasionally the cells lining the lumen of the acinus, and only these, showed some degree of differentiation to the normal adult cell with the clear reticular cytoplasm and basal nucleus. There is a close similarity between the hyperplastic and malignant cells and particularly between the hyperplastic acini and those of the intra-acinar type of carcinoma, and there is evidence of a gradation between one and the other (Figs. 8, 9, 10).

This hyperplasia occurred not only in prostates with carcinoma but in normal glands, and particularly in those with benign hypertrophy. The amount of hyperplasia varied. Table IV shows in the whole series of 142 its incidence and extent. It can be calculated from this table that the
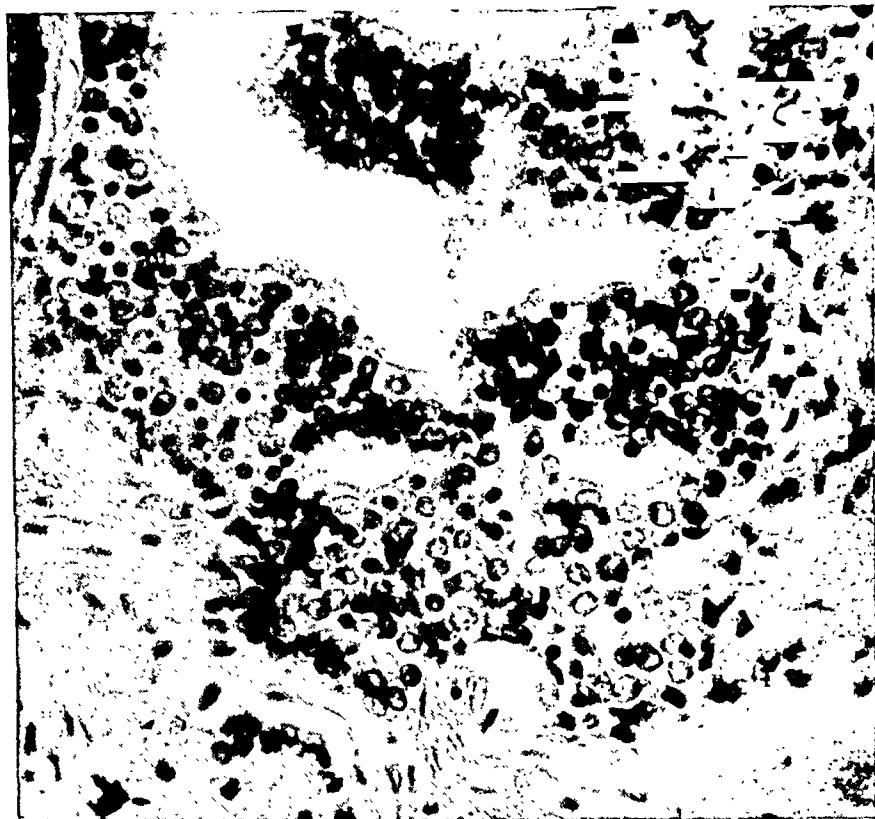
FIG. 8.—Hyperplastic acinus in which cells have broken through the stroma propria into adjacent muscle. × 400.

composed of acini without a basal layer of epithelial cells, whereas in the other four no specific histological features were detected.

Discussion

It may be concluded that microscopic - carcinoma of the prostate is common and occurs particularly beneath the capsule in the posterior lobe in men over the age of 60 years. It infiltrates the capsule and perineural lymphatics early.

Such a high incidence of microscopic carcinoma is difficult to interpret. It is well known that some small growths in the prostate may give rise to widespread metastases (Turnbull and Worthington, 1908), and it would seem that in these latent cases there may be some factor, possibly hormonal, that is restricting growth and dissemination.

incidence of this hyperplasia does not increase with age but that there is a definite increase in its incidence and extent both in benign hypertrophy and carcinoma and particularly with the latter.

It is most significant that in 41 of the 45 prostates with this hyperplasia the change occurred in the posterior lobe and was actually confined to this lobe in 27 of them. The lateral lobes were involved in 17 and the middle in one. In four cases this hyperplasia took part in " adenoma " formation, one of these being in the posterior lobe.

In one of the prostates without the specific hyperplasia the carcinoma seemed to arise in an " adenoma "

FIG. 9.—Malignant cells, indicated by arrow, " budding off " a hyperplastic acinus and rupturing the stroma propria. (Note the change in nuclear appearance.) × 400.

TABLE IV

INCIDENCE OF HYPERPLASIA IN NORMAL AND ABNORMAL PROSTATES

Condition of Prostates		Normal			Benign Hypertrophy			Carcinoma		
Age Groups:		15–29	30–59	60+	15–29	30–59	60+	15–29	30–59	60+
Degree * of hyperplasia ...	—	8	38	2	0	17	27	0	1	4
	+	2	5	0	0	6	11	0	1	1
	++	0	2	0	0	0	6	0	0	3
	+++	0	0	0	0	0	1	0	1	6

* The extent of the hyperplasia, i.e., the number of acini showing this change, is indicated by degrees of −.

A slow rate of growth, however, does not detract from the clinical significance. Flynn (1946) describes a case of occult carcinoma of the prostate which took 12 years to become widespread and cause death.

Although the high incidence of microscopic carcinoma in the prostate is rather puzzling, it must be remembered that the prostate has been chosen for such a thorough histological investigation because of its convenient size. A high incidence of microscopic carcinoma in other organs might be revealed by a similar method of study.

The close connexion between benign hypertrophy and carcinoma of the prostate has not always been supported by other writers. Although Albarran and Hallé (1900) considered benign hypertrophy to be a precancerous condition, Moore (1935) thought that carcinoma was intimately associated with and probably derived from epithelial cells that had previously undergone senile change, and Rich (1935) that carcinoma arose more commonly from senile and atrophic glands compressed by hypertrophied areas. Muir (1934) was unable to find any predisposing cause except age, and Kahler (1939) found no connexion between carcinoma and any other condition. Objections to regarding benign hypertrophy as a precancerous condition are that it is very common, that carcinonoma rarely arises in an "adenoma," and that carcinoma

occurs more commonly in the posterior lobe which is seldom affected in benign hypertrophy. The finding of a distinctive hyperplasia, frequently in the posterior lobe, which may undergo malignant change and is common to both benign hypertrophy and carcinoma, provides a closer link between the two conditions. The changes seen are similar to those described in carcinoma of the breast by Muir (1941), who showed that in certain cases intra-duct or intra-acinar hyperplasia preceded neoplasia. Such a similarity was also shown by Cheatle and Wale (1929), who
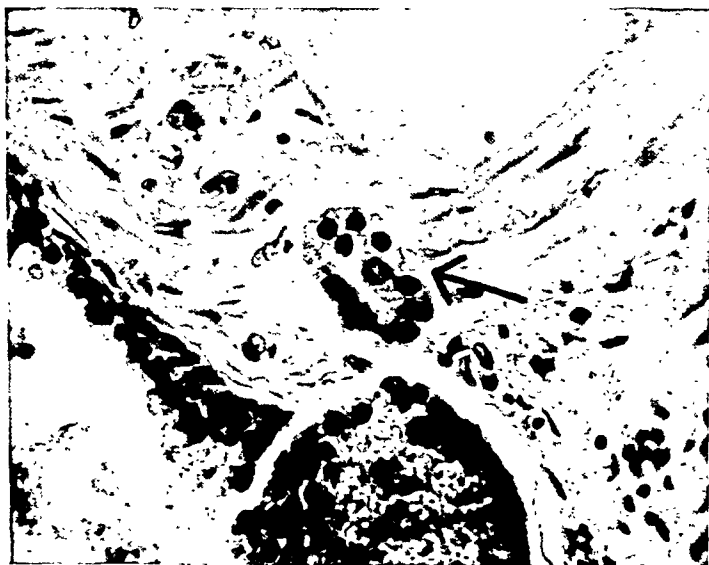
FIG. 10.—Small malignant acinus, indicated by arrow, appearing to have " budded off " from a hyperplastic acinus and ruptured adjacent muscle fibres. × 400.

believed that carcinoma of the prostate may be preceded by a " cystiphorous desquamative hyperplasia." No evidence was found that cyst formation in the prostate was preceded by desquamation, and it is not clear whether the hyperplasia described by Cheatle and Wale was similar to that seen in this study, but the ultimate conclusion appears to be the same—namely, that certain cases of carcinoma of the prostate are preceded by hyperplasia.

Summary

A detailed histological study of 142 prostates removed post-mortem was carried out and latent carcinoma found in 12%.

The criteria of malignancy adopted and the histology of the carcinomata are described.

Latent carcinoma was found most commonly beneath the capsule in the posterior lobe in men over 60 years of age. It was occasionally multifocal in origin.

Benign hypertrophy and latent carcinoma of the prostate are closely associated.

A specific form of hyperplasia is described which commonly occurs in, but is not confined to, benign hypertrophy and sometimes becomes neoplastic.

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THE USE OF PANCREATIC EXTRACT AS A GROWTH STIMULANT FOR *C. DIPHTHERIAE*

BY

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Gordon (1945), investigating the influence of various components of serum on the growth of *C. diphtheriae*, used pancreatic extract (liq. trypsin co.) and extract of gastric mucous membrane (" pepsin ") to prepare serum digests. He found that these extracts themselves, particularly the former, had a markedly stimulating effect on the growth of *C.diphtheriae* and that they retained this power even after the digestive enzyme content had been inactivated by heat. The active principle in the pancreatic extract is water-soluble, passes readily through a collodion membrane, and is thus of low molecular weight.

Further investigations were undertaken to determine whether pancreatic extract could with any advantage be incorporated in some of the blood tellurite media commonly used for the isolation of *C. diphtheriae*. The heated blood tellurite agar of Anderson *et al.* (1931) prepared with low temperature meat extract gives excellent type differentiation but is at a disadvantage when certain haematin-sensitive *mitis* and *gravis* strains (Glass, 1937 ; Robertson, 1943) are encountered. This difficulty is overcome by using unheated instead of heated blood (Glass, 1937). Hoyle's (1941) tellurite medium, though not suggested specially for haematin-sensitive strains, permits their growth as it contains lysed unheated blood in a Lemco agar plate. Hoyle claimed rapidity of diagnosis but admitted that type differentiation was inferior to that on McLeod's medium. Johnstone and Zinnemann (1943) used an unheated blood tellurite mixture and low temperature meat extract, and showed that the low temperature meat extract in McLeod's medium is largely responsible for good type differentiation. Hoyle's medium was very much improved by the substitution of low temperature meat extract for Lab. Lemco broth, and Johnstone and Zinnemann's medium prepared with Lab. Lemco broth produced growth little better

than that on Hoyle's original medium. The preparation of low temperature meat extract involves a somewhat complicated technique and may prove of considerable difficulty·under certain conditions. It was thought that the addition of pancreatic extract to blood tellurite media might prove a useful substitute for low temperature meat extract.

Method

The pancreatic extract chiefly used for its trypsin content was liq. trypsin co. It is made by extracting pancreatic glands with dilute alcohol acidified with phosphoric acid. The trypsin-containing-fraction is precipitated from this solution by alcohol and is then dissolved in 1% phosphoric acid, and an equal volume of glycerine is added to obtain a product of a more or less constant trypsin content. A pancreatic extract made for us without glycerine being added, and one made in the laboratory by the method described for the preparation of Hartley's broth (Mackie and McCartney, 1948) gave equally satisfactory results. Pancreatic extracts may vary in strength compared with liq. trypsin co., and the optimum concentration for incorporation in the medium varies accordingly. Before use the liq. trypsin co. was adjusted to *p*H 7.6 and boiled for five minutes to sterilize and incidentally inactivate the digestive enzyme fraction. Some reduction in the growth-promoting properties of the extract occurred if it were sterilized by autoclaving, but it still remained quite potent. A slight precipitate appeared on boiling the extract, and as its removal also resulted in some diminution of growth-stimulating properties the precipitate was retained. The boiled extract was added to the media to be investigated just before pouring.

The cultures of *C. diphtheriae* used were a number of stock *gravis*, *mitis*, and *intermedius* strains showing the typical characteristics described by McLeod (1943), and also some haematin-sensitive strains.

Results of the Experiments.—One to 10% boiled liq. trypsin co. adjusted to *p*H 7.6 was added to (1) unheated lysed blood tellurite agar with Lab. Lemco base (Hoyle); (2) heated blood tellurite agar (McLeod);

(3) a medium made with unheated blood tellurite mixture (Johnstone and Zinnemann). Media Nos. 2 and 3 were prepared both with Wright's broth (1933) and with low temperature meat extract. On all media reinforced with certain concentrations of pancreatic extract *C. diphtheriae* colonies showed markedly increased growth, particularly as regards the size and depth of single colonies. This was especially noted with *gravis* and *mitis* strains. The concentrations of pancreatic extract required to produce the optimum effect varied with the different media and appeared to be related to the presence or absence of unheated blood and also particularly to the presence of low temperature meat extract (see Table).

TABLE

Medium	Optimum Concentration of Pancreatic Extract (%)
Heated blood tellurite agar prepared with Wright's broth	5.0
McLeod's tellurite medium	2.5
Hoyle's medium	2.5
Johnstone and Zinnemann's medium	1.0

Any marked addition of pancreatic extract above these percentages may have an inhibitory effect and produce smaller *gravis* and *mitis* colonies, though *intermedius* colonies do not appear to be so much affected.

The increase of the size of the colony on blood tellurite media containing pancreatic extract was also associated with better type differentiation, and this was particularly striking on media, such as Hoyle's, prepared without low temperature meat extract. On the latter the differentiation between *gravis* and *mitis* strains may be confusing, as *mitis* colonies often appear rougher and duller, and *gravis* colonies smoother, than on other media. The addition of pancreatic extract to the medium promotes a smoother and shinier *mitis* colony and at the same time emphasizes the dullness and roughness of *gravis* colonies (see Figs. 1–6). Also pancreatic extract further emphasizes type characteristics even on media prepared with low temperature broth, producing round, shiny, confluent *mitis* and daisy-head *gravis* colonies.

Pancreatic extract has no influence in promoting growth of haematin-sensitive strains of *C. diphtheriae* on heated blood media, but when it is incorporated in media permitting their growth it has a stimulating effect, though somewhat less than on normal *gravis* and *mitis* strains.

The effect of the incorporation of pancreatic extract in blood tellurite media on other organisms frequently encountered is obviously of some importance, as any marked stimulation of their growth may nullify its advantage in the recognition of *C. diphtheriae* from

routine throat swabs. Culture plates of Hoyle's medium containing up to 5% of boiled liq. trypsin co. were inoculated with strains of *C. hofmanni, B. subtilis,* streptococci, *H. influenzae,* and yeast. Of these *C. hofmanni,* streptococci, and *B. subtilis* showed a slight increase in the size of the colony, though much less than the increase of *C. diphtheriae* colonies. *B. subtilis* colonies also presented a more mucoid appearance but showed no tendency to spread. The slightly stimulating effect on these contaminants was probably due to the glycerine content of liq. trypsin co., as it did not occur if a glycerine-free pancreatic extract was used.

Cantani in 1910 found that *C. diphtheriae* grew exceedingly well on a glycerine blood agar medium. This fact was later made use of by Clauberg (1935-6), and his Media II and III contain a proportion of glycerine (1933). Our further experiments showed that the glycerine content of liq. trypsin co. played no appreciable part in the stimulating effect on the growth of *C. diphtheriae,* and this appeared to be due solely to the pancreatic extract.

The addition of 5% pancreatic extract to Wright's nutrient broth also had a stimulating effect on growth of *C. diphtheriae,* with emphasis on type characteristics. Thus, of 16 stock *gravis* strains which during subculture over several years had lost the capacity to form pellicles on Wright's broth, nine showed typical coarse *gravis* pellicles when grown in this medium reinforced with pancreatic extract. *Mitis* strains grown in it exhibited a surface ring of bacterial growth adherent to the wall of the test tube, a characteristic first described by Hettche (1935; 1935-6). Some *mitis* strains occasionally developed pellicles, but these were soft, quite unlike *gravis* pellicles, and on shaking the test tube a uniform turbidity was produced in contrast to the coarse, floccular deposit from *gravis* pellicles.

Discussion

Certain workers abroad report difficulties in placing a considerable number of their *C. diphtheriae* strains within the three main types described by McLeod (1943). Although this may be due in part to the peculiarities of these strains, it is possible that the absence of low temperature broth in the tellurite media is partly responsible. It is an interesting speculation whether the active principles in low temperature broth and in pancreatic extract are similar and, if so, whether these are also present in other tissues. An effect similar to that of pancreatic extract is obtained with extract of gastric mucous membrane, and it is noteworthy that Gordon (1945) also reported the presence of a potent growth stimulant for *gravis* and *mitis* strains of *C. diphtheriae* in an aqueous extract of yeast. Recently a growth factor for Gram-positive bacteria has been described by Darzins (1948), though its characteristics do

not suggest that this factor is identical with the principle active in pancreatic extract.

As the growth-stimulating substance in pancreatic extract is still active after the tryptic enzyme has been destroyed by heat, the effect here described is not comparable to that in media such as Hartley's broth (1922), the preparation of which depends on active tryptic digestion. Pancreatic extract in the form of active trypsin was used in tellurite media containing serum by Douglas (1922) and Allison and Ayling (1929) for the purpose of neutralizing an antitryptic power of serum postulated by A. E. Wright (1915). In the light of the present observation the improved growth observed on these media may in part have been due to the heat-stable component of pancreatic extract rather than to the effect ascribed to the tryptic enzyme.

Owing to the present exceedingly low incidence of clinical diphtheria in the Leeds area it has not been possible to test tellurite media containing pancreatic extract in the routine examination of swabs, but the experimental results obtained seem to indicate its use being well worth a trial especially where low temperature broth media are not available.

Apart from its uses in bacteriological diagnosis pancreatic extract may be of importance in the production of *C. diphtheriae* toxin, as the latter is to some degree dependent on the extent of pellicle formation which, as shown, is stimulated by pancreatic extract.

Summary

Extract of pancreas as prepared for use in tryptic digestion contains a thermostable component which markedly stimulates growth and also emphasizes colonial type characteristics of *C. diphtheriae*.

Maximum stimulation of both growth and type characteristics is dependent on an optimum concentration of pancreatic extract.

The incorporation of pancreatic extract in tellurite media is of practical value for the isolation and typing of *C. diphtheriae*. Contaminants usually present in throat and nasal swabs are not stimulated to any extent.

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FIG. 1.—*C. diphtheriae gravis* on Hoyle's medium. × 14.2.
Strain 577/7 incubated for 48 hours.

FIG. 2.—*C. diphtheriae gravis* on Hoyle's medium + 2.5%
pancreatic extract. × 14.2. Strain 577/5 incubated
for 48 hours.
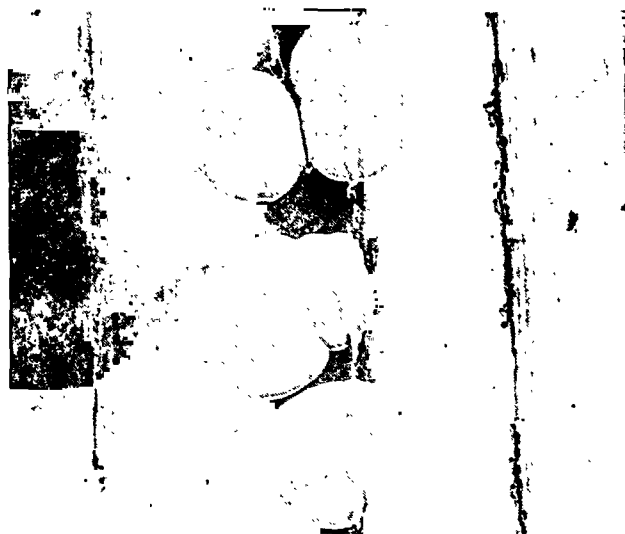
FIG. 3.—*C. diphtheriae mitis* on Hoyle's medium. × 14.2.
Strain Y 523 incubated for 48 hours.

FIG. 4.—*C. diphtheriae mitis* on Hoyle's medium + 2.5%
pancreatic extract. × 14.2. Strain Y 523 incubated
for 48 hours.
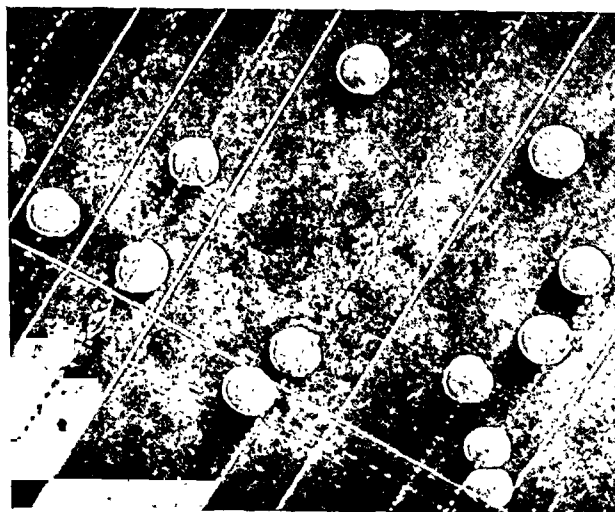
FIG. 5.—*C. diphtheriae intermedius* on Hoyle's medium.
× 14.2. Strain 214 incubated for 48 hours.
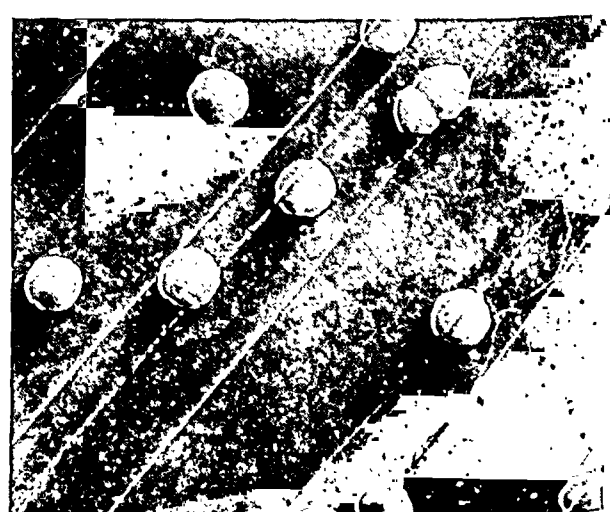
FIG. 6.—*C. diphtheriae intermedius* on Hoyle's medium
+ 2.5% pancreatic extract. × 14.2. Strain 214
incubated for 48 hours.

ERRORS IN THE ESTIMATION OF STREPTOMYCIN IN SERUM

BY

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In the report on specific laboratory tests in streptomycin therapy of tuberculosis (Medical Research Council, 1948) a subcommittee of the Medical Research Council recommended two techniques for the estimation of streptomycin in serum. One of these was a method involving serial dilutions in a serum-glucose–phenol-red indicator system on a waxed slide, followed by incubation in capillary tubes. The other was an agar diffusion technique carried out in small diameter tubes. An attempt has been made to assess the errors involved in using these two techniques under laboratory conditions similar to those in which they will actually be used.

Plan of Experiment

During the past two years estimations of streptomycin in serum have been made at Colindale using the capillary tube technique, whereas at the Postgraduate School of London the agar diffusion method has been used. One of us (D.A.M.) prepared a series of thirty sera containing known quantities of streptomycin. Each serum was divided into two portions, one of which was sent to Colindale and assayed by the capillary tube technique (H.D.H.), and the other was assayed at the Postgraduate School of London by the agar diffusion method (S.H.M.). At the beginning of the experiment it was agreed that the range of streptomycin concentrations should be between 4 and 50 μg./ml., which is the usual range found in patients on intramuscular therapy. No other information about the experimental plan was communicated till the end of the experiment, and no comments were passed on the results obtained.

The thirty sera tested were prepared as follows. Serum was obtained from healthy people and from ambulant patients attending for a variety of complaints. None of them was being treated with any

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form of chemotherapy. Serum from a different person was used for each specimen. The specimens were made up by adding appropriate quantities of an aqueous solution of streptomycin hydrochloride (Merck, Batch 594) containing 1,000 μg./ml. Portions of the same solution were used for preparing the standards at the Postgraduate School of London and at Colindale. Three specimens were prepared on the same day and estimated together. Estimations were done on ten different days over a period of a month. On every day one each of the specimens was made up within each of the subranges 4–8.99, 9–19.99, 20–50 μg./ml. Altogether there were thus ten specimens within each of these subranges. The concentrations within each subrange were selected at random from all possible concentrations using a table of random numbers. The labelling of the specimens was also strictly at random.

Techniques

Capillary Tube Method (Medical Research Council, 1948).—The indicator medium contained 1 part horse serum, 1 part 10 per cent glucose, 2 parts water, and phenol red as an indicator. To this was added sufficient of a 24-hour broth culture of *Klebsiella pneumoniae* III to make a dilution of 1 in 50. Serial dilutions were made in drops of saline on waxed slides with a Pasteur pipette, and to each of these an equal volume of the indicator medium was added. Capillary tubes were filled from the drops and incubated horizontally with the ends unsealed for 24 hours at 37° C. A standard containing 16 μg./ml. was prepared in horse serum and set up in the same way at the same time. The reading was determined by comparison with the standard.

Agar Diffusion Method (Mitchison and Spicer, 1949).—One per cent nutrient agar (Japanese) with a *p*H adjusted to 7.8–8.0 contained in 1-oz. screw-capped bottles was seeded with an 18-hour broth culture of *Staphylococcus aureus* (N.C.T.C. 7361), to make a final dilution of 1 in 1,000. The agar was distributed with a Pasteur pipette into a series of tubes 7 to 8 cm. long, 3 mm. in internal diameter, and sealed at one end. The sera were pipetted on to the tops of the agar columns. Standards were prepared in human

serum containing 4 and 50 μg./ml. Six tubes were set up for each standard and four for each test serum. After 24 hours' incubation at 37° C. the depth of the zone of inhibition is measured to the nearest 0.1 mm. using the traversing millimetre scale of a microscope with a crosswire in the eye-piece. The average of the readings from each serum was squared. The square of the zone of inhibition was linearly related to the log of the streptomycin concentration. A graph was plotted, one axis being $\log_{10}$ streptomycin concentration and the other axis the square of the zone of inhibition. The two points given by the readings of the standards were plotted and a straight line drawn between them. From this line the $\log_{10}$ of the streptomycin concentration in the test sera could be read off.

Results

Details of the results obtained are given in Table I. The results were converted into $\log_{10}$ value and the estimated values of streptomycin concentration were plotted against the true values (Figs. 1 and 2). A regression line of estimated on true values was calculated. From the resulting analysis the following information was obtained:

1. A deviation of the mean of the estimated readings from the mean of the true readings would indicate a general tendency to either under- or overestimate the streptomycin concentrations. In neither of the methods was there a systematic tendency of this nature.

2. The regression coefficients were tested for deviations from 1.0. There was no real difference in the case of the agar diffusion method (regression co-efficient 1.04101 ± 0.06370). In the case of the capillary tube method the value found differed significantly from 1.0 (regression coefficient 0.58485 ± 0.14509). This indicates a systematic error tending to overestimate low values and underestimate high values of streptomycin concentration. Thus, apart from experimental variation, where the estimated value is 4 μg./ml., the true value will be about 69 per cent less, and where the estimated value is 50 μg./ml. the true value will be about 86 per cent greater.

3. An estimate of the error of both techniques was obtained. The question requiring an answer is: At any estimated level of streptomycin concentration what are the limits within which the true value lies? For nineteen out of twenty assays the true value will lie within the fiducial limits that have been plotted in Figs. 1 and 2. Some representative values in numerical terms are given in Table II.

TABLE I

RESULTS OF ASSAYS OF STREPTOMYCIN IN SERUM

Day	True value (μg./ml.)	Estimated value	
		Capillary tube method (μg./ml.)	Agar diffusion method (μg./ml.)
1	8.79	4	8.9
	11.63	16	13.2
	26.74	32	23.4
2	7.85	32	7.2
	10.65	11	10.0
	20.16	21	17.3
3	5.50	16	4.7
	15.20	20	12.0
	48.48	64	47.9
4	8.90	5.3	8.0
	10.18	11	10.0
	21.34	11	20.9
5	4.48	21	4.8
	16.87	43	18.6
	45.49	64	53.7
6	6.33	6	8.3
	8.37	8	9.8
	20.01	8	22.4
7	7.50	16	7.4
	19.25	32	24.6
	44.92	48	72.4
8	4.14	12	4.5
	10.39	8	11.2
	37.47	24	37.2
9	4.22	16	4.1
	10.57	8	9.5
	42.52	24	45.7
10	8.83	8	8.1
	18.91	16	18.2
	43.50	32	45.7

TABLE II

FIDUCIAL LIMITS OF TRUE VALUES

Estimated value μg./ml.	Agar diffusion method μg./ml.	Capillary tube method μg./ml.
50	63–34	737–11.7
32	41–22	343– 5.5
16	21–11	115– 1.84
8	11–5.7	32– 0.58
4	6.0–2.8	9.8– 0.15

FIG. 1.—*Agar Diffusion Method.* The $\log_{10}$ of the estimated values of the streptomycin concentration have been plotted against the $\log_{10}$ of the true values. A regression line has been fitted to the points and the fiducial limits ($P = 0.05$) for the true values plotted. The vertical lines represent the streptomycin concentration in the two standards used.

$Y = 1.1343 + 1.04101 X$

LOG$_{10}$ OF TRUE VALUES OF STREPTOMYCIN CONCENTRATION

$Y = 1.12164 + 0.58455 X$

LOG$_{10}$ OF TRUE VALUES OF STREPTOMYCIN CONCENTRATION
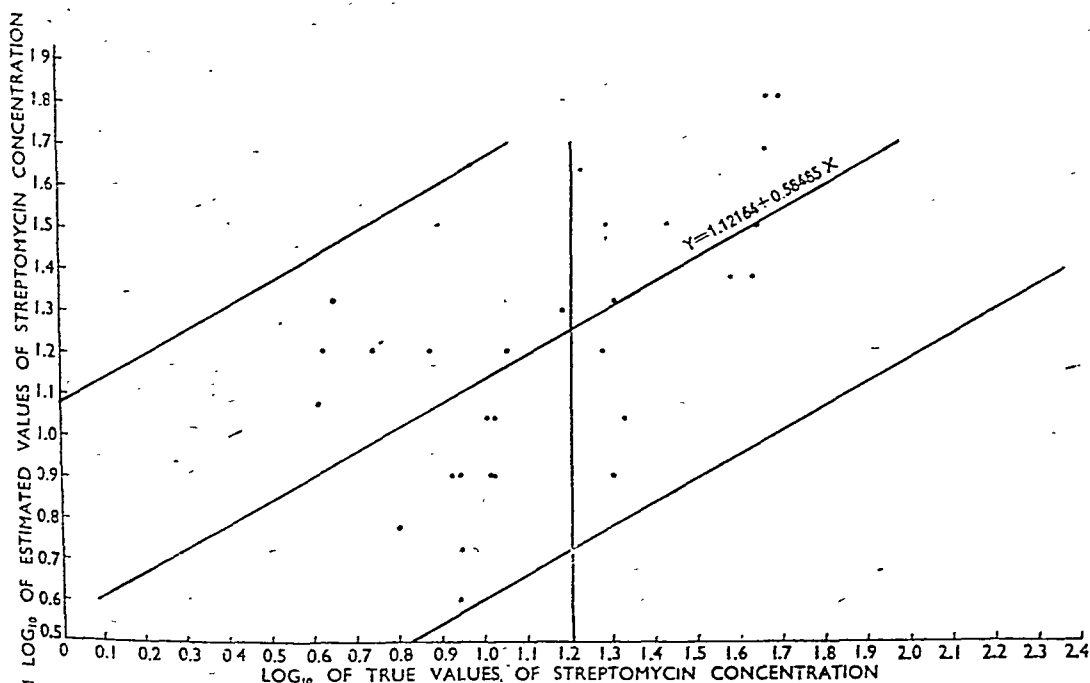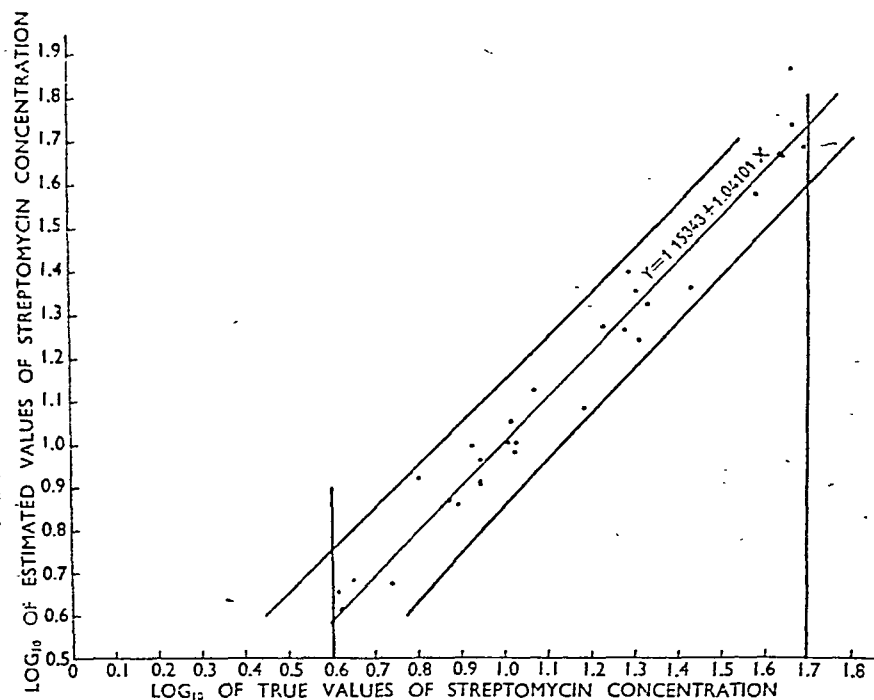
FIG. 2.—*Capillary Tube Method.* The $\log_{10}$ of the estimated values of the streptomycin concentration have been plotted against the $\log_{10}$ of the true values. A regression line has been fitted to the points and the fiducial limits ($P = 0.05$) for the true values plotted: The vertical line represents the streptomycin concentration in the standard used.

Discussion

From internal evidence (Mitchison and Spicer, 1949) the standard error (in logarithm units) of a typical assay using the agar diffusion technique was given as ±0.0422. Owing to the use of sera derived from different individuals this figure should be multiplied by 1.73, resulting in an estimated standard error of ±0.0730. This figure is in good agreement with the estimates of the standard error obtained in this experiment. For instance at the mean of the estimated values the standard error was ±0.0660.

It is clear that the errors involved in the capillary tube method are very large, and are particularly great when the readings on the test and control differ by more than one or two tubes. It might be advisable to set up standards at more than one streptomycin concentration with the test sera to be assayed. Even so the result of one estimation alone is highly unreliable. In view of the systematic tendency to underestimate high values and overestimate low values, even numerous readings will give fallacious answers unless several different standards are set up. The reasons for this systematic error are not clear. May and others (1947) indicate that organisms are inhibited by a higher concentration of streptomycin when serum water is diluted in saline. In the technique used here, serum dilutions are made in saline so that proportion of serum in the drops falls with each successive dilution. This might explain our findings. It might be advisable to carry out a similar experiment diluting the test serum in normal serum rather than in saline, in which case the composition of each drop would be constant. We feel that it is important that an estimate of error should be made of any new technique of assay which is tried.

Notes on the Statistical Analysis.—Let the $\log_{10}$ of the true values be x and of the estimated values y. A regression line fitted to the values will have the form $y = \bar{y} + b (x - \bar{x})$. For the two sets of data we have, where s_i is the standard error of i, the results shown in Table III.

TABLE III

STATISTICAL ANALYSIS OF CAPILLARY TUBE AND AGAR DIFFUSION METHODS

					Capillary tube method	Agar diffusion method
$\bar{x}$	..	..	..	..	1.14257	1.14257
$\bar{y}$	..	..	..		1.21640	1.15343
s_y^2	..	..	..	..	0.065896	0.004058
s_x	..	..	..	..	±0.04687	±0.01163
b	..	..	..	..	0.58485	1.04101
s_b	..	..	..	..	±0.14509	±0.06370
Deviation of b from 1.0	t				2.861	0.842
28 degrees of freedom	P				0.01–0.001	0.5–0.4
Deviation of $\bar{y}$ from $\bar{x}$	t				1.575	0.934
28 degrees of freedom	P				0.2–0.1	0.4–0.3

The fiducial limits for x were calculated from

$$s_x^2 = \frac{s_y^2}{b^2} + \frac{(y' - \bar{y})^2 \, s_b^2}{b^4}$$

Summary

Known concentrations of streptomycin within their range 4–50 μg./ml. were made up in 30 specimens of serum and assayed by a capillary tube technique and an agar diffusion technique. The capillary tube technique was shown to underestimate high concentrations of streptomycin and overestimate low concentrations. Fiducial limits of the true concentration expected from any estimated value within this range have been calculated.

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STUDIES *IN VITRO* ON THE MATURATION OF ERYTHROBLASTS IN NORMAL · AND PATHOLOGICAL CONDITIONS

BY

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During the past few years we have been studying the mechanism of the maturation of erythroblasts by observing the survival *in vitro* of bone marrow cells. By this means the maturation of erythroblasts of adults and children, both normal and pathological, has been followed in physiological and various experimentally modified conditions. We believe that this method furnishes more accurate information on the maturative activity of normal and pathological bone marrow than morphological studies of biopsy specimens recorded by means of maturation curves or indices.

Method

Our method consists in the introduction of 1–2 ml. of aspirated heparinized bone marrow into small Carrel flasks, the marrow being obtained from the sternum in adults and from the diaphysis of the long bones in children. Equal amounts of heparinized plasma from the same patient and of Ringer, or, preferably, Tyrode solution, are added. A small quantity of the suspension is immediately removed for erythrocyte and reticulocyte counts. The latter are performed on films stained with brilliant cresyl blue. We determine the percentage of reticulocytes relative to the erythrocytes, and the maturation curve according to Heilmeyer (dividing the reticulocytes for the sake of convenience) into three groups only : (1) Group " O," corresponding to orthochromatic erythroblasts ; (2) Group " A," or reticulocytes, Types I and II ; (3) Group " B," or reticulocytes, Types III and IV of Heilmeyer. Erythroblast counts are also made on films stained with May–Grünwald–Giemsa. Both the percentage relative to the erythrocytes and the maturation curve (after Pontoni) of the erythroblasts are determined.

Immediately after the removal of the specimens required for counting the culture flasks are incubated at 37° C. Further specimens are removed for counting at intervals of six or twelve hours, up to the 96th hour or later. All these operations are performed under conditions of bacteriological sterility.

After counting. the percentages of erythroblasts and reticulocytes are converted into absolute values by using the known absolute erythrocyte counts, and, for ease of inspection, the data are charted, times being represented on the abscissae and cell counts on the ordinates. Besides the graphs showing the absolute values. it is also useful to draw curves of relative values, such as the maturation curve after Pontoni (1936) and the reticulocyte curve after Heilmeyer (1942).

Under our experimental conditions the maturation of erythroblasts and reticulocytes continues, but their proliferation comes to an untimely end. We can therefore evaluate the approximate maturative rhythm of erythroblastic tissue from a study of our maturation curves.

It would, however, be a mistake to assume that the events occurring *in vitro* are necessarily identical with those in the living organism. Conditions *in vitro* differ fundamentally from those in the body in the following respects. The conditions of both anabolism and catabolism are dissimilar. There is an absence of nervous and hormonal control (with the exception of hormonal or other factors contained in the culture medium). In cultures mature elements persist, whereas in the living organism they enter the circulating blood. The normal mechanism of the destruction of blood is absent.

In experiments carried out by this technique normal and pathological erythroblasts show distinct differences in their behaviour, although they live in an identical medium. This fact permits the conclusion that any such differences observed *in vitro* depend on intrinsic differences in cellular constitution. Moreover, by means of this method we are able to modify the culture medium, and thus to study directly the effects of chemical. physical, or hormonal agents on bone marrow cells.

Results

A. Normal Bone Marrow.—This section summarizes the results in adults and children of various ages of Astaldi and Bernadelli (1945, 1946), and of Astaldi and Reggiani (1946a,-b).
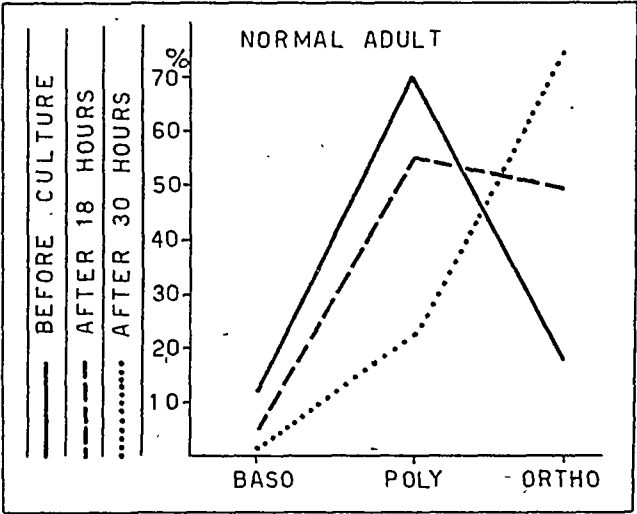
FIG. 1.—Maturation of erythroblasts in culture of normal adult bone marrow. Note progressive relative decrease of basophil and polychromatic, and increase of orthochromatic, forms.

The maturation curves (Fig. 1) show that, while polychromatic erythroblasts predominate in the early stages of culture, their number declines from the 18th to the 20th hour, at which stage it equals that of the orthochromatic cells. Also basophilic erythroblasts are becoming less numerous at the same time. Towards the thirtieth hour orthochromatic cells begin to predominate.

The absolute number of orthochromatic erythroblasts increases at an early stage, sometimes as soon as the sixth hour, and reaches a maximum at 12 to 18 hours, more rarely at 24 hours. Later it declines progressively to fall below the initial level at 72 hours.

Coincident with the decrease in the orthochromatic erythroblasts, a moderate increase in the most immature reticulocytes (Group "A") is observed.

The fall in the number of reticulocytes of Group "A" is followed by a rise in those of Group "B," which are generally considered as approaching full maturity; later, however, these also disappear (Fig. 2).

These results show that maturation is possible *in vitro*: basophil erythroblasts become polychromatic, then orthochromatic, and eventually, by loss of their nuclei, reticulocytes. Finally, the reticulocytes mature. The duration of the different phases of maturation is: 18–24 hours for the basophil–polychromatic, and 12–24 hours for the polychromatic–orthochromatic stages; about 24 hours for the transformation from orthochromatic erythroblasts to reticulocytes of Group "B," and 24–36 hours for the final maturation to adult erythrocytes. The cycle of complete maturation thus requires about 100 hours *in vitro*.

Orthochromatic erythroblasts mature partly to reticulocytes of Group "A," but also to those of Group "B," or directly to adult erythrocytes. When stained with brilliant cresyl blue, they can often be seen to contain fragments of granulo-filamentous substance, in consequence of an asynchronism between nuclear and cytoplasmic maturation; these elements, following the elimination of their nuclei, will later appear as reticulocytes of Group "B" or as adult erythrocytes.

The work of Tolentino (1947) on erythroblasts and reticulocytes in children of different ages gives results more or less similar to those observed in adults, as far as infants and children of pre-school

FIG. 2.—Maturation of reticulocytes in culture of normal adult bone marrow: Group "O," orthochromatic erythroblasts; Group "A," reticulocytes with reticular granulo-filamentous substance; Group "B," reticulocytes with filamentous and granular substance.
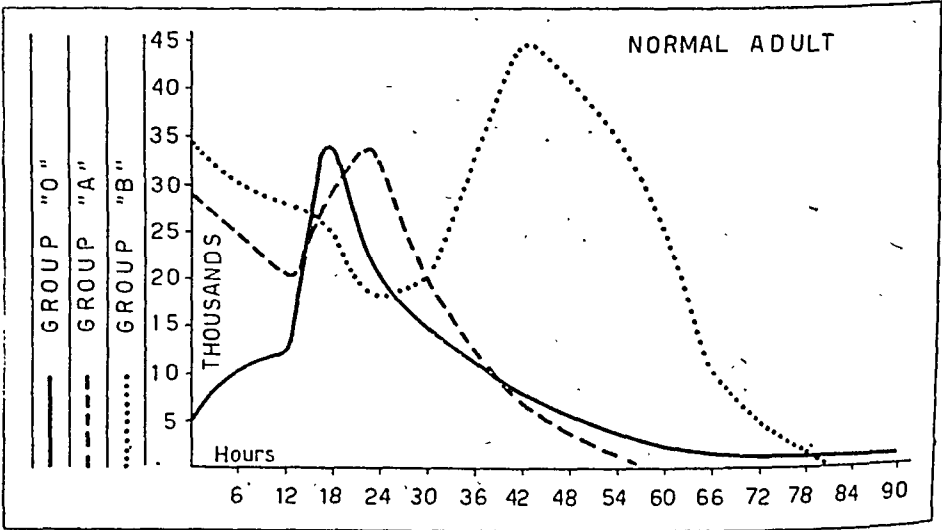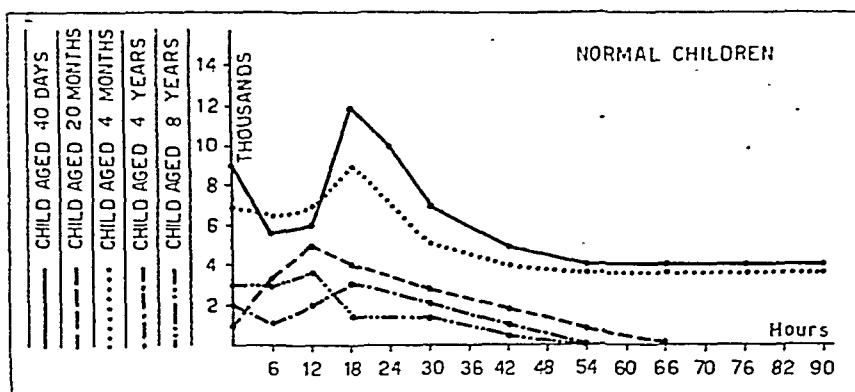
FIG. 3.—Maturation of orthochromatic erythroblasts in bone marrow cultures from normal children showing also delay in extrusion of nucleus in neonates. Normal maturation in older children.

and school ages are concerned. In neonates up to one month old, there is a normal maturation of the reticulocytes, accompanied by a delay in the maturation of the orthochromatic erythroblasts: values of 25% of the observed maximum are still found at the end of the experiments (Fig. 3).

This slow maturation might perhaps explain the great frequency of erythroblastaemia at this age when there is an abnormal peripheral demand for erythrocytes, as in haemolysis, haemorrhage, or cyanosis. It thus becomes unnecessary to postulate the presence of an extramedullary haemopoiesis.

B. Erythroblastoses.—In this group of diseases we have chosen cases of Cooley's anaemia, congenital haemolytic disease, and infantile kala-azar, all of which are accompanied by erythroblastosis and haemolysis.

Cooley's Anaemia.—The first investigations of Fieschi and Astaldi (1946) were followed by those of Astaldi and Reggiani (1946a, b), and unpublished ones by Astaldi and Tolentino. These have shown that erythroblasts mature normally down to the orthochromatic stage. In more serious cases, as in that published by Astaldi and Reggiani, a tendency

to a delayed extrusion of the nucleus of orthochromatic erythroblasts has been noticed (Fig. 4, Case I). This difficulty of maturation was still evident in circulating erythroblasts (Fig. 5): it seems therefore that these erythroblasts are useless for the production of adult circulating erythrocytes.

We have called the phenomenon of delayed extrusion of the nucleus "nuclear deficiency" for morphological reasons: it may, however, originate in a more complex protoplasmic deficiency. It has proved particularly evident in the most serious cases: in slighter cases, marrow erythroblasts have, on the other hand, shown a behaviour not far from normal (Fig. 4, Cases III and IV).

It is a noteworthy peculiarity that erythroblasts which do not eliminate their nuclei show a complete maturation of the cytoplasm: in fact, while getting older, they lose those remnants of the basophil substance which can normally be demonstrated in many orthochromatic erythroblasts by suitable stains like brilliant cresyl blue.

Haemolytic Disease of the Newborn.—We have so far investigated two cases of this disease following in vitro the behaviour of medullary and cir-
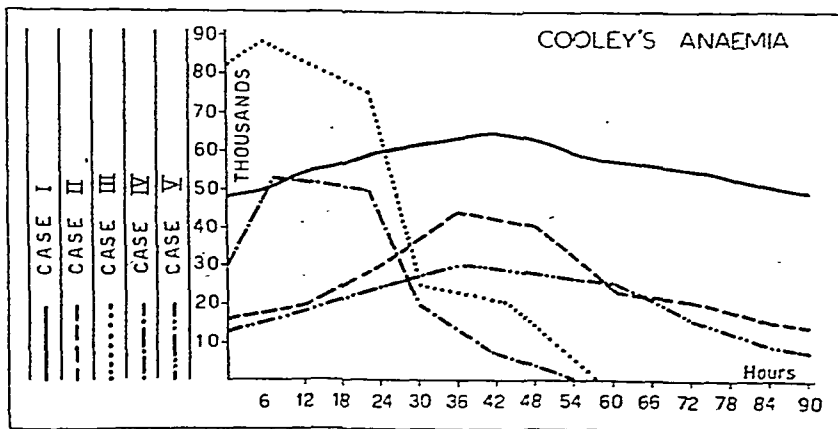
FIG. 4.—Maturation of orthochromatic erythroblasts in bone marrow cultures from patients with Cooley's anaemia, and delayed extrusion of nucleus in some cases.

culating erythroblasts and reticulocytes. As in the case of Cooley's anaemia we have also been able to extend our study to the circulating elements (Astaldi and Tolentino, 1946). This is obviously impossible in normal subjects.

Fig. 6 shows that we found a decrease in the number of erythroblasts to one-fifth of the initial value in one case, and to one-third in the other, both towards the 48th hour. The reticulocytes of Group "A" in the first case diminished progressively, disappearing completely as early as the 30th hour, while those of Group "B" had disappeared at 70 hours. In the second case the corresponding figures were 48 and 84 hours.

On the whole we may say that erythroblasts mature normally, compared with the values obtained in normal neonates. Further, circulating erythroblasts may mature completely, as we observed *in vivo* in one of the two cases, in which there was a remarkably high number of erythroblasts in the peripheral blood. It follows that the passage of nucleated elements into

the blood does not in this disease prejudice the production of adult functioning erythrocytes. None the less, the less intense and slower maturation in one of our two cases, compared with the other, reproducing the clinical and haematological differences, appears.to show that there may be a superadded marrow failure, accounting for a delayed maturation.

Leishmaniasis.—We have observed that maturation proceeds as usual from basophil to orthochromatic erythroblasts, and that the reticulocytes, too, mature normally. On the other hand, there appears to be an obstacle to the extrusion of the nuclei in orthochromatic erythroblasts, when these are compared with normal elements (Fig. 7). This difficulty does not seem to be connected either with the inhibitory action of the spleen, or with the number of parasites present in the bone marrow: even in such cases it seems to be a function of the clinical and haematological condition
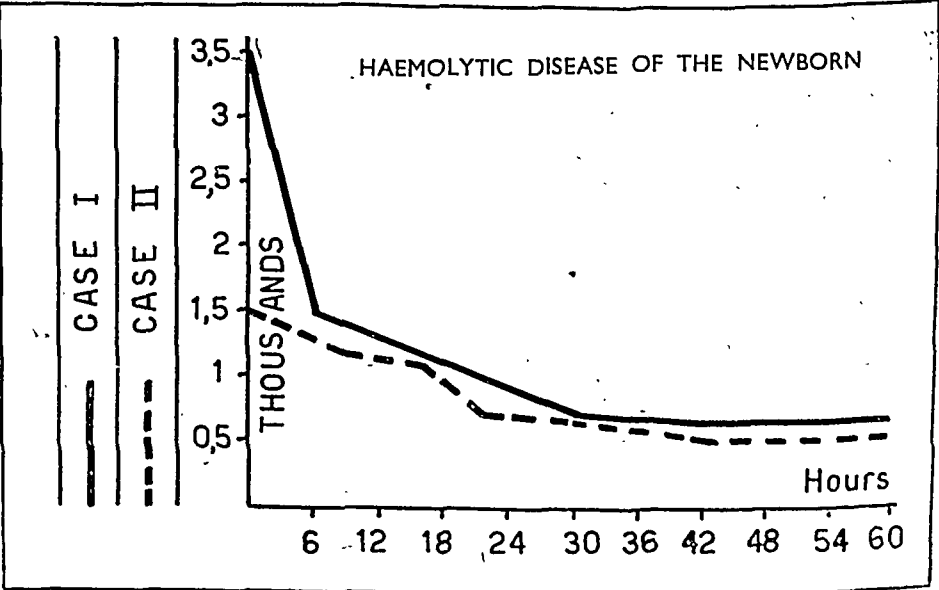
FIG. 6.—Normal maturation of orthochromatic erythroblasts in cultures from two children with haemolytic disease of the newborn.

of the patient, and a result of exhaustion of the bone marrow in consequence of the severe and long-continued haemolysis. Failure of ortho-chromatic erythroblasts to extrude their nuclei ("nuclear deficiency") which has been evident in

all our cases of Leishmaniasis, may possibly lead to their accumulation in the bone marrow; if so, an increased percentage of orthochromatic erythro-blasts in maturation curves may actually be an indication of a check in maturation.

FIG. 7.—Maturation of orthochromatic erythroblasts in bone marrow cultures from four children with leishmaniasis showing delay in extrusion of nucleus. (The delay was greatest in the most severe cases.)

FIG. 8.—Maturation of erythroblasts in bone marrow cultures from a case of pernicious anaemia showing delayed maturation from basophil to ortho-chromatic stage, corrected by addition of liver extract.
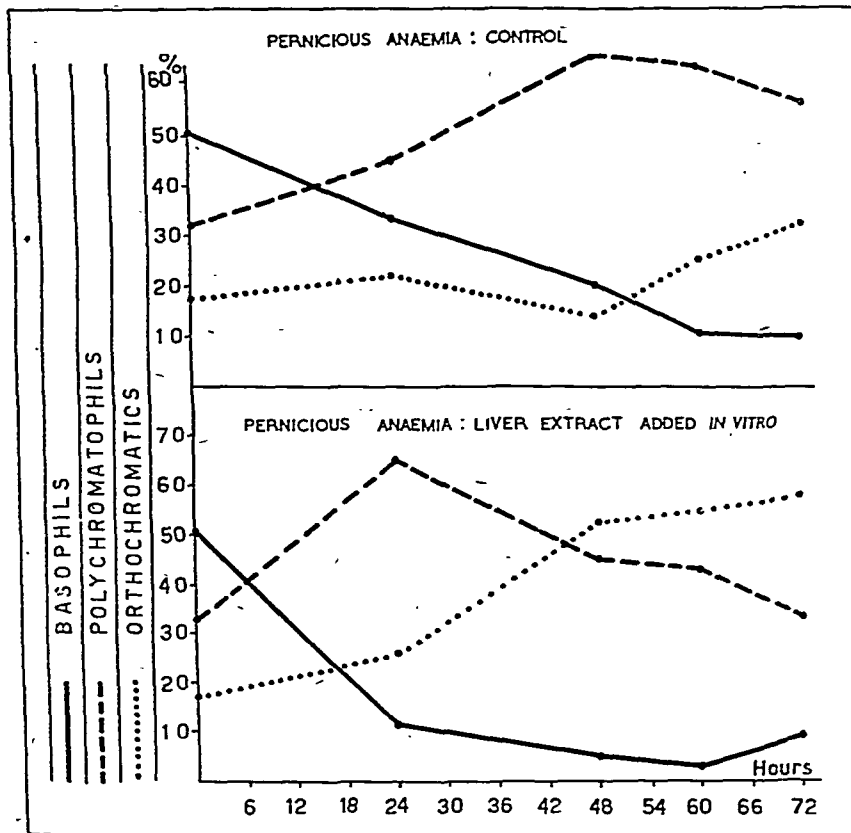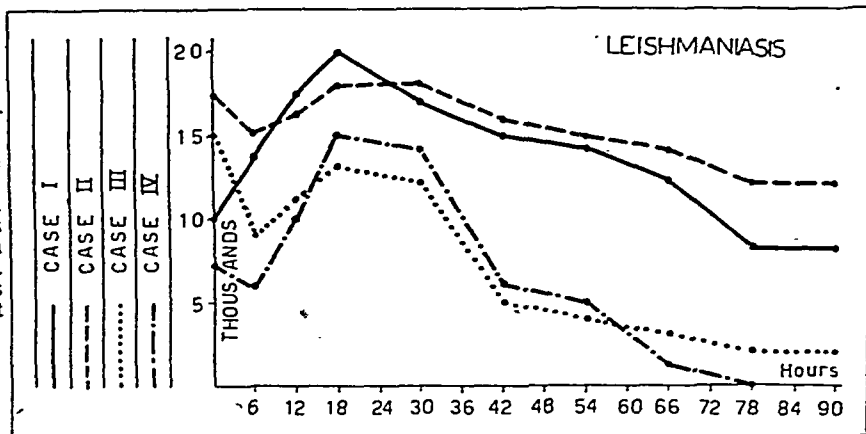
C. Pernicious Anaemia. — Astaldi, Baldini, and Frugoni (1946a, b, 1948) studied the explanted bone marrow of patients with per-nicious anaemia, com-paring the maturative rhythm of megalo-blasts with that of normoblasts. In this way they were able to demonstrate that the evolution from the basophilic to the orthochromatic stage is slower for the megaloblast than for the normoblast, the whole process requir-ing not less than three

days for the former, but not more than two days for the latter (Fig. 8).

D. Effects of Various Agents.—Our method is suitable for the study of temperature and various agents on cells surviving *in vitro*.

Temperature.—The influence of temperature on the process of maturation of erythroblasts and reticulocytes has been studied in several experiments. In these it was found that, within certain limits, an increase of temperature above 37° C. accelerates the process, but at temperatures above 42° C. there appear signs of cellular damage, such as lysis. A decrease of temperature inhibits maturation, which practically ceases below 20° C.

Liver Extracts.—Astaldi, Baldini, and Frugoni have demonstrated an acceleration in the maturation of megaloblasts in pernicious anaemia, following the addition of liver extracts to the culture medium. The evolution from the basophil to the orthochromatic stage appeared to be quicker in the presence of extract than in the controls (Fig. 8).

Spleen Extracts.—Tolentino and Lombroso (1947) found a delay in the maturation of normal erythroblasts following the addition to the cultures of pathological human spleen extract from a case of lipoidosis. A delay in the maturation of erythroblasts was also observed in another case, in which both the cells and spleen extract were obtained from the same patient with Cooley's anaemia.

Niacin.—Niacin may have an accelerating influence on the maturative rhythm of megaloblasts surviving *in vitro*, as shown by the work of Astaldi and Baldini (1948). In contrast to the action of liver extracts, however, the addition of niacin does not lead to a normoblastic transformation of the megaloblasts.

Colchicine.—Astaldi, Mauri, and Tolentino (1948) have described a delaying action of colchicine on the maturation of normal bone marrow cells *in vitro*.

Summary

The method we have described and adopted has proved useful in the study of the maturation of erythroblasts and reticulocytes. The following applications have been of practical value. Standard values of the duration of maturation *in vitro* for normal subjects at various ages have been obtained.

We have been able to point out that some apparently sound and authoritative theories in the dynamics of haematology may stand in need of revision: thus our demonstration of possible delays at the orthochromatic stage is in contrast to the accepted view (Fieschi, 1946, and Rohr, 1940) that inhibition of the maturation of red cell precursors always leads to a " shift to the left " in the medullary formula. The significance of maturation curves or indices becomes thereby less clear cut.

We have been able to show that marrow failure may express itself in " nuclear deficiency " of orthochromatic erythroblasts, and to formulate a hypothesis explaining the various phases of the clinical and haematological picture found in Cooley's anaemia, haemolytic disease of the newborn, and leishmaniasis. It has been possible to demonstrate the influence on the maturation of erythroid cells of various factors applied *in vitro*.

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DEXTRAN AS A MEDIUM FOR THE DEMONSTRATION OF INCOMPLETE ANTI-Rh-AGGLUTININS

(PRELIMINARY REPORT)

BY

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Dextran is a polysaccharide which is formed by Leuconostoc mesenteroides and has the composition (glucose-H_2O) n. A structural formula has been suggested by Levi and others (1942). Grönwall and Ingelman (1945) and Ingelman (1947) have shown that a partly hydrolysed 6 per cent solution of dextran in normal saline, of molecular weight approximately 100,000, is a suitable plasma substitute, and it has been used as such in Sweden for some years.

Experimental Observations

A woman with anaemia due to a ruptured ectopic pregnancy had received 760 ml. of a solution of dextran just before the withdrawal of blood for determination of her Rh group by the rapid slide method. The cell suspension was made in the patient's own serum. It was known that the test sera used gave weak or no agglutination if the cells were suspended in serum that had been diluted with normal saline. In the present case the saline for dilution contained 6 per cent dextran, and strongly positive reactions were observed, the control (cell suspension only) showing no agglutination. The activity of incomplete anti-Rh-antibodies in a solution of dextran was therefore investigated.

Method.—Eleven sera with incomplete anti-Rh-antibodies with titres between 1:8 to 1:1,600 were titrated against fresh cells of type O R_1R_1 and O rr in: (1) normal saline, (2) normal human serum (a mixture of ten sera), and (3) the solution of dextran used as a plasma substitute.

The technique used was as follows: tubes measured 8 × 0.8 cm. and 0.25 ml. volumes were placed in each tube. The cell suspensions were made in the same media as the serum dilutions. The tubes were incubated for four hours at 37° C. They were gently shaken before reading and reactions were read by means of a hand lens. An irregular contour was not in itself considered evidence of a positive reaction.

Results.—Normal human serum and dextran had about the same enhancing effect on the incomplete agglutinins in the sera tested, and titres varied between 8 and 1,600. In saline medium agglutination was not observed beyond the serum dilution 1 : 2. The Rh-negative cells were not agglutinated.

The enhancing effect of dextran on these agglutinins vanished below a concentration of 2.5 per cent dextran (the dilutions of dextran made in normal saline).

The enhancing effect of dextran on incomplete anti-Rh-agglutinins was also observed if a small volume of serum dilution and cell suspension (about 0.01 ml. each) are incubated for three hours at 37° C. and the results read under the microscope. On such readings the cells in the control tubes often show a rouleaux-like arrangement, and if the salt concentration is increased to 1.5 per cent this pattern is more pronounced. If, on the other hand, the salt concentration is lowered, the cells are more evenly distributed, and at a concentration of 7 per cent dextran and 0.65 per cent NaCl no such pattern is observed although an enhancing effect is still retained.[*]

TABLE

TITRES AGAINST SHIGELLA BACILLI

	Normal saline	6% dextran
Chicken serum I ..	400	3.200
Chicken serum II ..	200	3.200

Sera from two chickens that had been immunized with Shigella bacilli were titrated in normal saline and in a medium of dextran (6 per cent dextran

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[*] Swedish dextran was used throughout the experiments, but a sample of English dextran was examined and showed the enhancing effect. However, this sample was more liable to give a rouleaux-like arrangement of the cells than the Swedish dextran.

in 0.9 per cent NaCl). The results (see Table) were read after incubation for twenty-four hours (four hours at 37° C. and then twenty hours at room temperature).

Sera from five patients suffering from infection with Brucella abortus were titrated against this organism using saline and dextran as media. The titre of the agglutinins was not increased by the use of the dextran medium.

Discussion

Dextran gives a perfectly water-clear solution that keeps at room temperature and is free from natural agglutinins. The solution is commercially available, is relatively inexpensive, and may be autoclaved. Its composition is well defined and can therefore be kept constant. It is unquestionably active in revealing the agglutinating effect of incomplete anti-Rh-antibodies. These qualities of dextran make it worth while investigating the conditions which are optimal for the test in regard to concentrations, specificity, activity, and avidity. Such a study must include trials of dextran of differing molecular size with varying concentrations of different electrolytes against a large panel of cells and sera. Until this has been done the activity of dextran cannot justly be compared with the activity of other materials used in the demonstration of the incomplete antibodies.

Dextran is a polymer of glucose, but glucose is not active in producing an agglutinating activity of incomplete anti-Rh-antibodies (Diamond and Denton, 1945). As the molecular size of dextran can be varied without difficulty, a new approach is opened to the study of the physico-chemical conditions that are required for the demonstration of agglutinins in incomplete anti-Rh sera.

The preliminary character of the present study is obvious, and it is not suggested that dextran should be used exclusively in Rh-testing on the basis of these observations.

Summary

Incomplete anti-Rh-antibodies were found to be active in a solution of dextran.

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HAEMOLYTIC ANAEMIA

BY

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(RECEIVED FOR PUBLICATION, MARCH 4, 1949)

The application of the technique described by Coombs and others (1945) has made it possible to demonstrate serological differences between cases of congenital and acquired haemolytic anaemia. This test determines the red cell agglutinating effect of serum from rabbits immunized against human serum globulins. Agglutination of the sensitized cells with a rabbit anti-human-globulin serum is taken to mean that the red cells have adsorbed an immune antibody globulin from the plasma ; this adsorbed globulin reacts with the antiglobulin rabbit antibody causing agglutination. Boorman and others (1946) demonstrated that washed red cells from patients with acquired haemolytic anaemia were agglutinated with rabbit anti-human-globulin serum, whereas similarly treated cells in seventeen cases of congenital haemolytic anaemia were not. Evans and others (1947) found that the red cells of two patients with acquired haemolytic anaemia were readily agglutinated by an immune rabbit serum containing an anti-human-globulin antibody. Moreover, the antibody adsorbed on the sensitized red cells could be eluted and readsorbed on to normal group O cells.

The antibody has been shown to be lipoidophilic, but has never been identified as due to any specific antigen. Under the title of " symptomatic haemolytic anaemia " many cases in the older literature are attributed to such causes as syphilis, tuberculosis, streptococcal septicaemia, paratyphoid fever, and cirrhosis of the liver ; and more recently haemolytic anaemias have been described in association with Hodgkin's disease, sarcoma of the spleen, reticulo-endotheliosis, leukaemia, sarcoid, carcinoma of the pancreas, and Salmonella infection.

This case of haemolytic anaemia is reported because of the observation that in a patient with a history strongly suggestive of syphilis the eluted antibody gave a positive Kahn reaction even though the serum Kahn and Wassermann were both negative. The subsequent improvement with antisyphilitic treatment affords further support to the specific aetiology of the haemolytic process.

Case Report

The patient was a male aged 69 years, and married. He was admitted to Addenbrooke's Hospital, Cambridge, on May 21, 1946, complaining of shortness of breath and general weakness since May, 1944. There was no relevant family history.

In 1922, in Marseilles, he had contracted a venereal infection which had resulted in a swelling on his penis. This was treated by a French doctor with local application of lotion, and the swelling disappeared. He was not given any injections, and there is no record of a Wassermann reaction having been done.

In 1936 he had a swelling of the right side of his scrotum, which was accompanied by a slight pain in the right testicle. He was treated with lotion, and the swelling disappeared.

In 1938 a friend noticed that he was jaundiced. He felt well, and he had neither nausea nor loss of appetite, but he thought he ought to remain in bed until the yellow colour had disappeared, which it did in three weeks. He had no recollection of the colour of his stools or urine at this time.

In 1939 he had a vesicular eruption on the right side of the face which lasted six weeks and left a residual scarring.

The present illness began in February, 1944, when for a brief period he felt tired and breathless. He saw his doctor, but the condition improved rapidly and he returned to work. By May, 1944, he found he was again getting short of breath when walking and he felt tired, especially after going upstairs, but these symptoms did not progress. He had had no tingling in hands or feet, and he had been free from cough, palpitations, and pain in the chest. He was given daily liver injections by his doctor. After these he noticed that his urine was red and soupy with sediment. This continued for the next nine to twelve months, but his urine had been orange-yellow during the year before admission. His stools for the same period had been yellow ochre in colour, bulkier than formerly, and offensive. He had not noticed any greasiness in them.

TABLE I
BLOOD EXAMINATION: MAY 21, 1946

Red cells	..	..	1.68 millions/c.mm.
Haemoglobin	..	..	6.15 g./100 ml.
M.C. Hb	..	..	36.6 γγ
M.C.V.	..	..	116.1 cμ
M.C. Hb conc.	..	..	31.5%
Reticulocytes	..	..	58%
Leucocytes	..	..	11.9 thousands/c.mm.

Differential W.B.C.
Neutrophils:

Myelocytes	..	..	None
Juvenile cells	..	..	12/c.mm.
Stab cells	..	..	477 ,,
Segmented cells	..	..	8,805 ,,
Eosinophils	..	..	595 ,,
Basophils	..	..	298 ,,
Lymphocytes (large)	..	..	665 ,,
Lymphocytes (small)	..	..	1,012 ,,
Monocytes	..	..	59 ,,
Platelets	..	..	487,000/c.mm.

Film :

Stippled cells	..	..	Moderate
Anisocytosis	..	..	Moderate
Poikilocytosis	..	..	Slight
Macrocytosis	..	..	Moderate
Abnormal cells	..	..	
Normoblasts	..	..	119/c.mm.

Arneth 1	..	..	..	5%
,, 2	..	..	..	11%
,, 3	..	..	..	29%
,, 4	..	↑ ..	..	35%
,, 5	..	..	..	20%

His doctor had noticed yellowness in his eyes for two years, but he himself had never noticed a similar colour in his skin during this period. He had had no bleeding from the gums or nose, and no bruising or spots, nor had he noticed any increased tendency to bleed. Since December, 1945, he had had a gnawing pain in the upper abdomen which came on two or three hours after meals. The pain began in the upper left quadrant and radiated over to the left side or occasionally over the middle upper abdomen. The pain was relieved by taking further food and by alkalis. Despite this new complaint he had felt that during the past five months his breathlessness and tiredness had been better.

He had increased frequency of micturition at night and a marked decrease in his exercise tolerance.

When examined, his conjunctivae were yellow and he had a mild icteric tinge to his skin. His mucous membranes were pale, but he had no angular stomatitis and his tongue was natural. His pupils were normal and reacted to light and accommodation, and no abnormality in the central nervous system was detected. His respiratory and cardiovascular systems were normal.

There was some distension of the abdomen without any tenderness, and the liver was enlarged from one to

two fingers' breadth below the costal margin and was not tender to deep palpation. His spleen, palpable below the costal margin, was firm but not tender. No other abnormal masses were felt, and no definite scarring could be detected on the penis. There were no significant lymph nodes palpable. He had a staphylococcal urinary infection which responded to treatment with sulphathiazole.

Special Investigations. — Electrocardiograms and radiographs of chest, abdomen, and bones were normal.

Urine.—Reaction, acid; specific gravity, 1019; sugar, *nil*; albumin, a trace; urobilin, strong positive. Deposit showed 50 red blood cells and 10 leucocytes per field, no casts or crystals, but profuse staphylococci.

Faeces.—Yellow ochre in colour; urobilin, 1.940 mg. per 100 g.

Blood.—N.P.N., 51 mg. per 100 ml.; total proteins, 6.4 mg.; albumin, 4.3 mg.; globulin, 2.1 mg.; fibrinogen, 0.46 g.; cholesterol, 140 mg.

Laboratory investigations showed that the van den Bergh test was a direct negative, but the quantitative test gave a reading of 4.0 mg. azobilirubin per 100 ml. of serum.

The results of a full blood examination are set out in Table I. The red cell distribution curve is shown
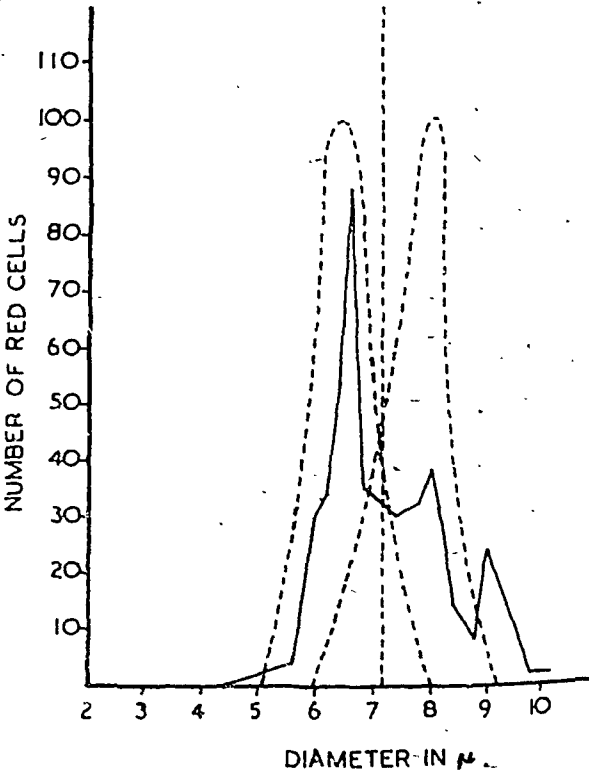
FIG. 1.—Red cell distribution curve. Arithmetic mean = 7.23 μ; standard deviation σ = 0.965 μ; coefficient of variation 13.3 per cent.
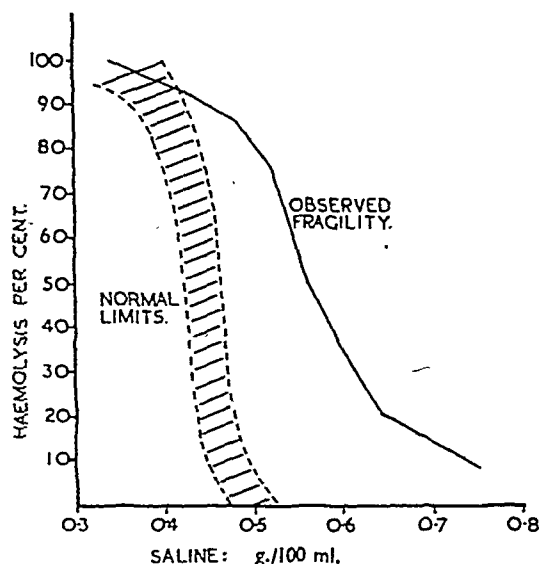
FIG. 2.—Quantitative fragility test.
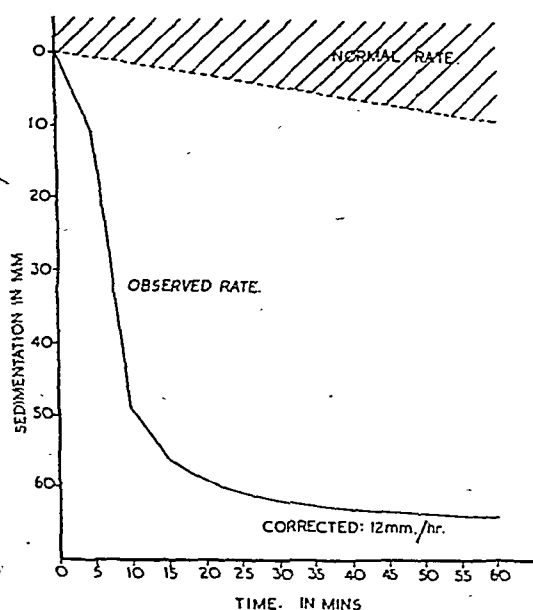
FIG. 3.—Sedimentation rate.

in Fig. 1. the results of the fragility test in Fig. 2, and the sedimentation rate in Fig. 3.

The Wassermann and Kahn reactions were negative, as also were the Donath-Landsteiner reactions. The blood was group B and Rh-negative. There was slight autohaemolysis when the patient's cells were in contact with his fresh serum at room temperature.

The patient's cells were washed three times in saline and added to rabbit antiglobulin serum ; this gave a strong agglutination ; that is, the antibody, γ globulin, was absorbed on the patient's cells.

The patient's serum (freshly centrifuged immediately after withdrawal) was absorbed by cells of the same group, which were then washed three times in saline. When these cells were added to rabbit antiglobulin serum there was slight agglutination only (that is, there was a trace of antibody in the serum).

The Kahn reaction, on the eluate obtained after preliminary heating of the patient's cells in contact with saline at 56° C. for 30 minutes, was positive + +. The Wassermann reaction remained negative. Controls with normal cells under similar conditions were negative.

Saline eluate obtained from the patient's cells was added to washed group O normal cells. These group O cells agglutinated with anti-globulin serum at 37° C. at room temperature, and at 4° C.

Progress.—The staphylococcal urinary infection improved with sulphathiazole. The patient was given a course of iodides and bismuth as it was considered that arsenic or penicillin might provoke a serious Herxheimer reaction and it was recommended that he be given a series of transfusions. For this purpose he went to Southmead Hospital in July, 1946. At this time his haemoglobin was 5.6 g. A transfusion of 1 pint of whole blood and 2 pints of packed cells raised his haemoglobin to 10.9 g., the transfusion being without reaction or abnormal incident. The transfused cells did not survive long in his circulation, and seven days later the haemoglobin level had again dropped to 6.7 g.

Twelve days after the first transfusion he was given a further transfusion of 3 pints of packed cells. again without reaction, and his haemoglobin level rose to 9.5 g., but a fortnight later was back at 7.4 g.

He had no further transfusions until September, 1946, when he had two, each of 1 pint of whole blood. The first pint raised his haemoglobin level from 6.0 to 7.0 g., and a week after the second pint his haemoglobin was 7.7 g. From that time onwards his anaemia improved and his haemoglobin level showed a steady rise up to 10.5 g. on Nov. 1, 1946 (Fig. 4). Every time blood was taken it was tested with rabbit anti-human-globulin serum, and it was always strongly agglutinated. He was seen at intervals subsequently, the last time being on May 26, when his haemoglobin was 12.7 g. and the red cell count 3.64 million per c.mm. of blood. On May 31 an estimation of the sternal bone marrow was made, and the results are summarized in Table II. Apart from some complaints of epigastric discomfort he was free from unpleasant symptoms and there had been no relapse. although the haemolytic process was still to some extent active.
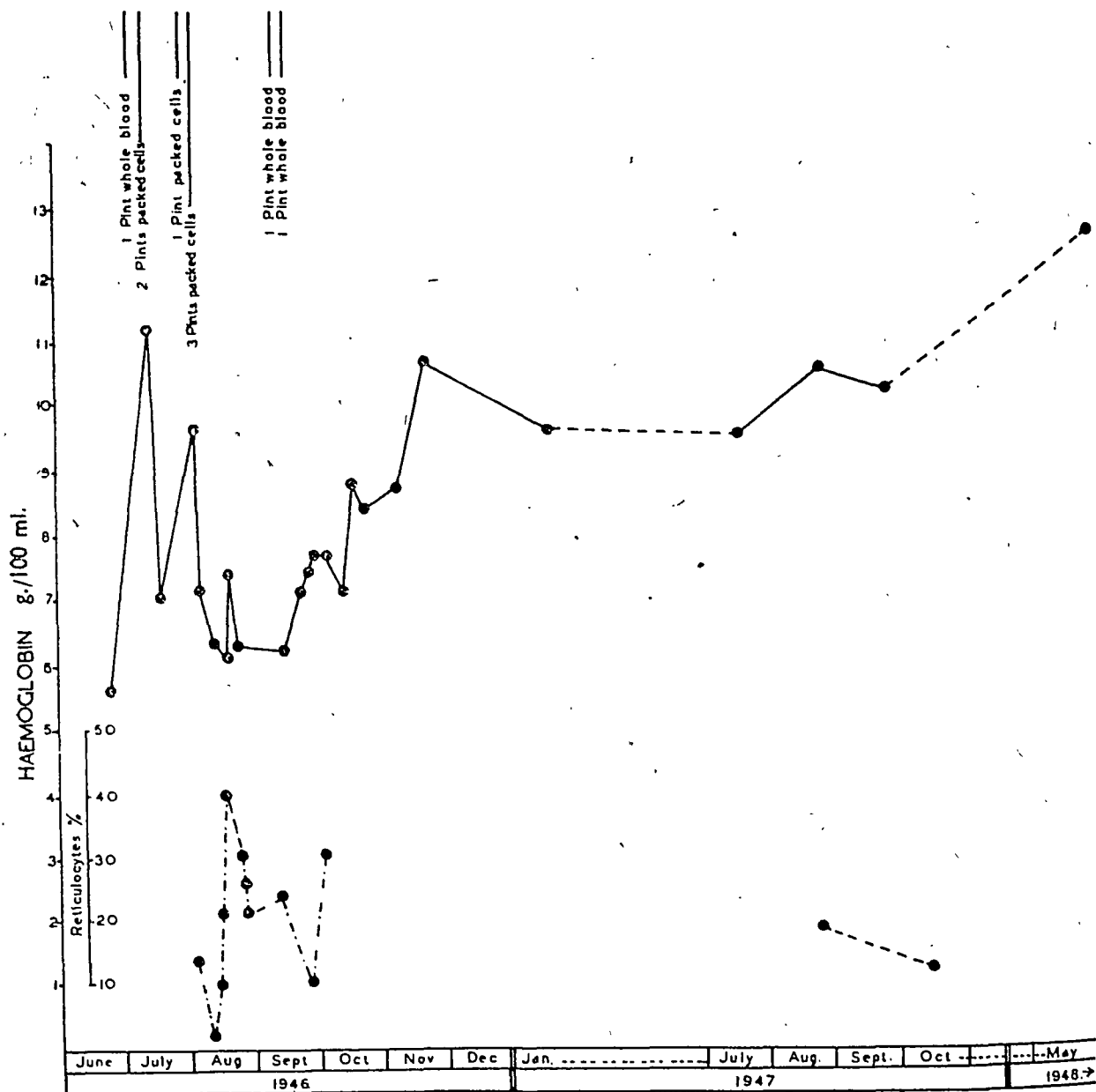
FIG. 4.—Haemoglobin levels: data supplied by Tovey and Hackett.

Discussion

This case had been previously diagnosed and treated for several years as refractory pernicious anaemia, though when the patient was admitted to Addenbrooke's Hospital the appearances were those of severe haemolytic anaemia with a macrocytic tendency, the bone marrow being of the macronormoblastic type. The red-cell distribution curve showed double peaks, the spherocytic cells being microcytic. The plasma, urine, and faeces were those of haemolytic anaemia. This clearly illustrates that the haemolytic syndrome may be a cause of macrocytic anaemia.

It is interesting to note that there was a staphylococcal urinary infection. This has been known to produce a haemolytic anaemia, but in this case the treatment of the urinary infection left the haemolytic process unaffected and it appears probable, therefore, that this infection was not the aetiological factor.

The history of venereal disease, and the fact that a lipoidophilic antibody is present in syphilis, suggests as a possible explanation of the haemolytic process that the invasion of the erythropoietic tissue by the *Treponema pallidum* resulted in the production of an antibody sufficiently specific to be selectively adsorbed on to the red cells and yet capable of producing a positive Kahn reaction. The fact that the antibodies were almost completely adsorbed on to the red cells may explain why the Wassermann and Kahn reactions were

TABLE II

STERNAL BONE MARROW: MAY 31, 1946

Total nucleated cell count	187,000/c.mm.
	%
LEUCOCYTE SERIES:	
Myeloblast	0.5
Promyelocyte	1.0
Neutrophil:	
Myelocyte	5.75
Juvenile	5.5
Mature	16.25
Eosinophil:	
Myelocyte	1.5
Mature	1.5
Basophil:	
Myelocyte	—
Mature	0.25
Lymphocyte	3.0
Monocyte	—
Plasma cells	1.75
NUCLEATED RED CELLS:	
Haemocytoblast ..	1.75
Pro-erythroblast ..	3.5
Early normoblast ⎱	30.0
Intermed. normoblast ⎰	
Late normoblast ..	21.25
Intermed. megaloblast ⎱	6.5
Late megaloblast ⎰	
Myeloid : erythroid ratio ..	1 : 2

both negative. Very little antibody was found in the serum, but it could be eluted readily from the red cells by means of saline at 56° C. The antibody could then be readsorbed on to heterologous red cells. This eluted antibody caused marked flocculation of the Kahn antigen as performed in the standard Kahn reaction. Controls using normal red cells under the same conditions produced negative results. Moreover, the washed red cells of the patient were strongly agglutinated with rabbit anti-human-globulin serum. Coombs (1947) examined the red cells from ten consecutive patients diagnosed as syphilitics with positive Wassermann reactions and no haemolytic anaemia. He found no evidence of red cell sensitization and he observed similar negative findings in three cases of pernicious anaemia and in one case of macrocytic anaemia associated with chronic nephritis.

It is unusual for an acquired haemolytic anaemia of such long standing and in a patient of this age to have responded to a few transfusions. Following the initial rapid haemolysis of the transfused cells there has been a continuous and steady improvement in the patient's condition. It is reasonable to assume that the antisyphilitic treatment played a significant role in this recovery.

Summary

A case of acquired haemolytic anaemia in an old subject with a probable syphilitic aetiology was found to respond to simple antisyphilitic treatment and a few transfusions. The syphilitic reaction was demonstrated by eluting the antibody.

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R

TECHNICAL METHODS

ESTIMATION OF *p*-AMINOSALICYLIC ACID IN BLOOD

BY

H. V. STREET

From the Crumpsall Hospital, Manchester

Owing to the increasing use of *p*-aminosalicylic acid in the treatment of tuberculosis, it was considered that a simple method employing an ordinary single-cell type of photoelectric colorimeter for the estimation of the drug in blood was desirable.

Several methods of determining it have been reported. Way and others (1948) use a modification of the Bratton-Marshall (1939) method for determination of sulphonamides. The method of Morris (1941) has been modified by Klyne and Newhouse (1948) who use 0.5 ml. blood, and by Spinks (1948) (using 0.1 ml. blood). These latter procedures involve anil formation with *p*-dimethylaminobenzaldehyde. Although Spinks's method apparently gives satisfactory results when the optical densities are measured on a spectrophotometer, it was found not to be very sensitive when a single cell direct reading type of photoelectric colorimeter was used.

Tennent and Leland (1948) outline a somewhat similar procedure to the method described below, in which they use two reactions. (1) The *p*-aminosalicylic acid in acid solution is coupled with diazotized *p*-nitroaniline, followed by the addition of sodium hydroxide to give a red colour ; and (2) a blue colour is produced when both the *p*-aminosalicylic acid and the *p*-nitroaniline are diazotized, followed by coupling in the presence of pyridine and the addition of sodium hydroxide. The effect of interfering materials is eliminated by reading both the red and the blue colours at 620 mμ, using the former as a blank for the latter.

The following method, which appears to be more suitable for use with the simple photoelectric colorimeter and when only small quantities of blood are available, has been devised.

Method

The proteins of blood are precipitated with trichloroacetic acid. *p*-Nitroaniline is added to the supernatant, and this and the *p*-aminosalicylic acid are diazotized with sodium nitrite. On the addition of sodium hydroxide coupling occurs and a purple dye is produced. Coupling presumably takes place initially at the *p*-position to the hydroxyl group of the *p*-aminosalicylic acid, which is probably followed by further coupling with more of the *p*-nitroaniline.

The stability of the colour is shown in Fig. 1. The intensity is at a maximum immediately after formation and decreases gradually on standing. This decrease, which is only slight after the solution has been
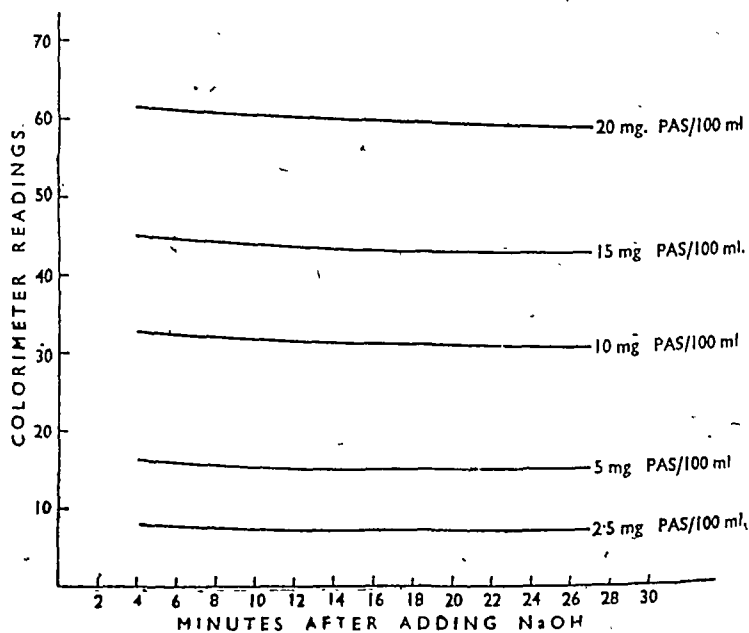
FIG. 1.—Stability of colour.

allowed to stand for 15 minutes, becomes smaller with decreasing concentration of *p*-aminosalicylic acid.

The following reagents were used:

1. 1.5N-trichloroacetic acid.

2. Sodium nitrite 0.5 per cent (w/v). This solution should be renewed after two weeks.

3. *p*-Nitroaniline 0.1 per cent (w/v) in 0.25 N-hydrochloric acid.

4. 2 *N*-sodium hydroxide.

5. *p*-Aminosalicylic acid standard. 11.4 mg. of anhydrous sodium *p*-aminosalicylate (or 13.8 mg. of the dihydrate) are dissolved in distilled water and made up to 100 ml. This solution is equivalent to 10 mg. *p*-aminosalicylic acid/100 ml.

0.2 ml. whole blood was measured into 3.2 ml. distilled water in a centrifuge tube. It was mixed well and allowed to stand for 3 minutes for the cells to

lake. Then 0.6 ml. trichloroacetic acid solution was added, mixed thoroughly, allowed to stand for 10 minutes, and centrifuged for 10 minutes. To 3 ml. of the clear supernatant liquid was added 0.3 ml. sodium nitrite solution and then 2 ml. of the *p*-nitroaniline reagent. This was mixed well and allowed to stand for 3 minutes, when 2 ml. sodium hydroxide solution was added. The colour was read after 15 minutes in a photoelectric colorimeter, using a green filter (Ilford No. 404). A reagent blank was prepared by using 0.2 ml. distilled water in place of the blood.

Standards were prepared from suitable dilutions of an aqueous solution of the dihydrate of sodium *p*-aminosalicylate and were used in the construction of a calibration curve (see Fig. 2). Since this curve is rectilinear, a single standard may be employed as an alternative and the following calculation used:

Concentration of unknown =

$$\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Concentration of standard}$$

A blank of the order of 1 mg./100 ml. is given by normal blood. The recoveries from whole blood are shown in the Table.

FIG. 2.—Typical calibration curve.

TABLE
RECOVERIES FROM WHOLE BLOOD

μg. Added	μg. Found	% Recovery
5	4.6	92
10	9.0	90
20	19.0	95
30	27.0	90
40	37.0	93
Mean recovery		92%

Summary

A simple method for the estimation of *p*-aminosalicylic acid in blood is described.

0.2 ml. of blood is required.

An ordinary single-cell photoelectric colorimeter is used.

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CONCENTRATION OF MEGALOBLASTS

AN AID IN THE DIAGNOSIS OF PERNICIOUS ANAEMIA

BY

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From Southlands Hospital, Shoreham-by-Sea

Typical megaloblasts, whose presence is so essential for the diagnosis of pernicious anaemia, are not often seen in the peripheral blood film. A method of concentrating these cells, therefore, should be of great assistance in the diagnosis.

The buffy coat in a haematocrit tube consists of white cells, which, in spite of their larger size, fall with less velocity than the smaller red cells. This consideration suggested that the bigger, nucleated red cells might be found in increased numbers in or immediately beneath the white cell layer, and this has proved to be the case. Incidentally, both macrocytes and microcytes are found concentrated in this area, thus exaggerating the typical pernicious anaemia picture. A further point of interest is the study of the white cells themselves, which in ordinary films are very scarce, owing to the generally occurring leucopenia. Cooke's Type I and II cells can thus be conveniently studied.

Method

Venous blood is collected in a tube containing Wintrobe's oxalate mixture, centrifugalized in a haematocrit tube, and the mean cell volume reading taken in the usual way. A Pasteur pipette, with an even tip, is then carefully inserted and part of the white layer, as well as the corpuscles immediately below it, aspirated. Enough plasma is normally obtained by the capillary action of the pipette for the resuspension of the cells. A small drop is placed on one end of a clean slide, and with a corner of another the cells are gently mixed and quickly spread in the usual manner. Three to six slides are made, as it will be found that the staining capacity, as well as the concentration of nucleated cells, varies between films. Megaloblasts at different stages of maturity will sometimes be found in different films. The films are allowed to dry and stained at once. It will be found that, in general, cells are well stained, little distorted, and easily recognizable. No rouleaux formation is seen. Sometimes, however, distortion is such as to prevent accurate determination of their structure, and it is advisable in such cases to repeat centrifugalization at a lower speed and for a shorter time.

In twelve cases examined, only one did not show typical megaloblasts in the peripheral film by the concentration method. Five minutes' search revealed on an average 12 nucleated erythrocytes. In contrast, only two cases showed megaloblasts without concentration. When sternal punctures were performed to complete a full examination this step proved superfluous in 11 cases. The daily use of the concentration method after the beginning of liver treatment showed that the megaloblasts survived for three or four days in the peripheral blood. Disappearance of these cells, therefore, is an indication of response to liver treatment. Although the method described gave good results in pernicious anaemia, there is no advantage in its use in hypochromic anaemias. One case of aleukaemic leukaemia was examined, and it seemed to be of value in the diagnosis.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology ; biochemistry ; haematology ; and morbid anatomy and histology.*

BACTERIOLOGY

Aureomycin, a New Antibiotic. Results of Laboratory Studies and of Clinical Use in 100 Cases of Bacterial Infections. FINLAND, M., COLLINS, H. S., and PAINE T. F. (1948). *J. Amer. med. Ass.,* 138, 946.

The authors summarize the results of laboratory studies on aureomycin and its use in 100 cases of bacterial infections at Harvard : 186 strains of various pathogenic bacteria were tested by dilution or "streak" plate methods. Haemolytic streptococci, pneumococci, gonococci, and meningococci were inhibited by 1 μg. per ml., while staphylococci and most Gram-negative bacilli were inhibited by 25 μg. per ml., except *Proteus vulgaris* and *Pseudomonas aeruginosa,* which required a concentration of 100 to 250 μg. per ml. for inhibition. No cross-resistance with penicillin, streptomycin, polymyxin, or bacitracin was found, nor was resistance to the antibiotic readily produced either *in vitro* or *in vivo.* No aureomycin-inhibiting substance could be shown to be produced by aureomycin-resistant strains. The size of the inoculum influences the concentration of aureomycin required for inhibition of growth in the tube-dilution method of assay. The dry powder was stable at room temperature, and in aqueous solution of pH 4 there was no loss of potency after 2 weeks at 4° C. Aureomycin is most active in acid solutions and is not adsorbed by the usual bacterial filters.

The antibiotic appears rapidly in the urine and is excreted for 2 to 3 days after a single oral dose of 0.5 g., the maximum concentration—up to 256 μg. per ml.— being present for 2 to 16 hours and appearing 4 to 8 hours after the dose. Plasma levels (estimated by unsatisfactory methods) of about 2 μg. per ml. were obtained with oral doses of 1 g. 6-hourly.

The clinical effect of oral administration of aureomycin in empirical dosage to 100 patients was estimated. Good results were obtained in 49 of 66 patients with gonorrhoea ; failures were most frequent in those receiving 1.5 g. or less daily, but in larger doses the results compared closely with those obtained with penicillin. Four patients with pneumococcal pneumonia and one with meningococcal septicaemia were treated, and a rapid response obtained. One of 5 typhoid-fever patients improved soon after treatment was begun, and in 3 cases the response was steady and gradual and all cultures from blood, urine, and faeces were negative for *Salmonella typhi* after 2 to 3 days. One of 3 patients with severe salmonella infection improved rapidly but the remainder died, as did a patient with *Bacterium coli* septicaemia. Aureomycin was used in 16 cases of long-standing urinary infection complicated by the presence of other lesions. Pyuria disappeared in half the cases, and in most there was symptomatic relief. In some, however, strains of *Proteus vulgaris, Ps. aeruginosa,* and *Bact. coli* resistant to aureomycin appeared. Favourable results were obtained in 2 cases of non-specific urethritis.

No sign or symptom of toxicity was found, save infrequent looseness of the bowels.

Aureomycin: A New Antibiotic with Virucidal Properties. I. A Preliminary Report on Successful Treatment in Twenty-five Cases of Lymphogranuloma Venereum. WRIGHT, L. T., SANDERS, M., LOGAN, M. A., PRIGOT, A., and HILL, L. M. (1948). *J. Amer. med. Ass.,* 138, 408.

The 25 cases of lymphogranuloma inguinale treated with aureomycin fall into three groups : (1) 8 patients with buboes ; (2) 3 with proctitis ; and (3) 14 with benign rectal strictures. In the first group, treatment consisted of a single daily intramuscular injection of 20 mg., with in 2 cases a single injection of 20 mg. into the bubo. In all cases there was a reduction in size of the bubo after 4 days, and in the 3 in which elementary and initial bodies had been found, these disappeared after about 9 days. For the second group of patients treatment was similar, the total doses being 60, 200, and 220 mg. respectively. After 4 days 2 had lost all tenderness and discharge, and in the third rectal bleeding ceased after 8 days. Of the 14 patients in the third group, 5 had previously undergone colostomy for obstruction and one had a recto-vaginal fistula. In 12 the stricture was so pronounced that not even the tip of the finger could be inserted at the time of the patients' admission to hospital. Treatment consisted of daily intramuscular injections of 10 or 20 mg. of aureomycin, with total doses ranging from 160 to 1,000 mg. There was cessation of rectal discharge and rectal bleeding in almost all, and of tenderness in all cases. There was no change in the stricture in 11 out of the 14.

The authors regard their results as encouraging, and recommend further large-scale investigations of this antibiotic.

Chemotherapy of Tuberculosis with Sulphetrone. CLAY, M. G., and CLAY, A. C. (1948). *Lancet,* 2, 180.

Forty-two patients with pulmonary tuberculosis were treated with "sulphetrone." Evaluation of sulphetrone treatment was based on the radiological picture, weight, erythrocyte sedimentation rate, sputum analysis, temperature range, and general condition of each patient before and after the course. Of the 42 cases, 7 were thought to have a good prognosis at the start of treatment, and all 7 patients improved; 5 patients in whom the prognosis was hopeless died. Of 8 patients with a fair prognosis 4 improved, 2 were unchanged, and 2 became worse. Of 22 with a poor prognosis 9 improved, 3 were unchanged, 4 became worse, and 6 died. Of the 42 cases 34 were sputum-positive before treatment, and 31 were still sputum-positive after treatment.

The dosage scheme was similar to that described by Madigan (see *Lancet,* 1948, 2, 174-180). In addition to the toxic effects he described, the present authors report

changes in the alkali reserve of the blood, particularly in patients with a low vital capacity. In 6 cases toxic reactions necessitated withdrawal of sulphetrone.

The authors believe that, while it could not be proved that the results obtained were due to sulphetrone, the evidence is suggestive enough to warrant further trials in selected cases.

Infection by Penicillin-Resistant Staphylococci. BARBER, M., and ROZWADOWSKA-DOWZENKO, M. (1948). *Lancet*, 2, 641.

This report deals with an extension of previous work by one of the authors ; it is shown that the incidence of penicillin-resistant strains of *Staphylococcus pyogenes* giving rise to infection in the hospital in which the work was carried out is increasing.

Specimens from 100 patients with staphylococcal infections were examined. From 59 patients penicillin-resistant strains of *Staph. pyogenes* were isolated ; from 39 all colonies were resistant and from 20 both resistant and sensitive strains were isolated. Comparison with previous results showed that the incidence of patients yielding penicillin-resistant strains had risen from 14.1% in 1946 to 38% in 1947 and 59% in 1948. It was found that 34 of the 59 penicillin-resistant strains were originally isolated from 48 mixed cultures, whereas only 25 penicillin-resistant strains were isolated from 52 pure cultures. All the 59 resistant strains produced penicillinase, and strains showing a minor degree of resistance were not encountered.

Streptomycin sensitivity was tested in 97 strains : 55 were penicillin-resistant, 13 were penicillin-sensitive strains isolated in association with 13 of the resistant cultures, and 29 were penicillin-sensitive strains isolated from cases yielding no penicillin-resistant colonies. All 42 penicillin-sensitive and 53 of the 55 penicillin-resistant strains showed a sensitivity to streptomycin similar to that of the Oxford staphylococcus.

It is considered that the widespread use of penicillin causes resistance in staphylococci in two ways : either a strain acquires resistance to penicillin or naturally resistant organisms, originally few, may survive while sensitive organisms are destroyed. In the present study it was found that, as regards penicillin-sensitive strains, 4 patients had had previous treatment with penicillin but 37 strains came from patients who had had no previous penicillin treatment. In the case of resistant strains, 29 came from cases with previous treatment and 30 from cases without such treatment.

Weil's Disease. Analysis of 195 Cases in England. BROOM, J. C., and ALSTON, J. M. (1948). *Lancet*, 2, 96.

The authors investigated 195 cases of leptospirosis diagnosed serologically in their laboratories between 1940 and 1946. Any degree of agglutination of *Leptospira icterohaemorrhagiae* by blood serum they regarded as suspicious, but when a titre is 1 in 300 or less confirmation is sought.

Analysing the occupations of the patients they distinguished between bathers (5%) and those working in water (7%) on such work as bridge repairs, because in the latter infection through the conjunctiva and mouth seemed unlikely. Of the cases 95.6% were in males. The jaundice rate (90%) and the case-fatality rate (22%) were both high, possibly because all patients were those requiring admission to hospital. Evidence is adduced that many cases of leptospirosis escape recognition.

Four Cases of *Leptospira canicola* Infection in England. A Symposium. LAURENT, L. J. M., NORRIS, T. ST. M., STARKS, J. M., BROOM, J. C., and ALSTON, J. M. (1948). *Lancet*, 2, 48.

The authors report 4 cases of *Leptospira canicola* infection occurring in England. They point out that, though canicola fever has been reported in man from most European countries, hitherto only one case has been fully investigated in England. The disease is very widespread among dogs all over the world. The 4 cases here detailed presented signs of meningitis with fever, a macular rash, and conjunctivitis. There were obvious changes in the cerebrospinal fluid. The diagnosis was obtained by demonstrating a high agglutination titre against *L. canicola* in the patients' plasma, and the evidence tabulated leaves no reasonable doubt about the correctness of this diagnosis. It is suggested that the possibility of leptospiral infection should be considered in all cases presenting signs of lymphocytic meningitis.

Jos. B. Ellison.

The Treatment of Typhoid Fever by the Use of Vi Anti-typhoid Bacteriophages. A Preliminary Report. DESRANLEAU, J. (1948). *Canad. J. publ. Hlth.*, 39, 317.

Not all strains of *Salmonella typhi* are sensitive to phage Type II, and Archambault and the author found that the need for carrying out preliminary sensitivity tests on the infecting strain before propagating Type II bacteriophage, a process occupying some days, delayed the commencement of treatment unduly. To minimize this delay a mixture of Types I to IV was prepared ; *in vitro* this polyphage was found to attack all Vi forms of *S. typhi* and also to keep for long periods in the ice box. Theoretically, therefore, this mixture should serve for the treatment of all cases of typhoid, provided the infecting strains are in the Vi form.

The polyphage was used in the treatment of 20 cases of typhoid fever, the dosage and method of Knouf *et al.* being followed. Reactions similar to those described by the same observers were noted ; transitory rigor with some increase in pyrexia occurred some hours after injection and was followed by a rapid fall in temperature to subnormal but, within 24 to 48 hours, the temperature returned to normal and the " typhoid aspect " disappeared. Five patients were treated in the early septicaemic stage (presumably during the first week) ; their blood cultures became negative " immediately " and no organisms were recovered from the dejecta. The results were variable among patients whose treatment commenced only when *S. typhi* had been isolated from the stools. More than half the cases responded in the same striking way as did the early cases, but the remainder required one or two additional doses of the polyphage " because of various complications." All of the patients recovered. The author concludes that intravenous injection of Vi-antityphoid bacteriophages yields good results during the early stage of the disease, and if given at a later stage affords effective protection in most cases, although the organism may be excreted for some time (3 women became carriers).

Caronamide and Penicillin. Serum Levels in Human Beings, Following Multiple Doses of the Drugs. MEADS, M., LONG, R. V., PACE, S. H., and HARRELL, G. T. (1948). *J. Amer. med. Ass.*, 138, 874.

The effect of caronamide given by mouth 4-hourly for 3 days on the serum penicillin levels of 17 patients was studied. None of them was over 60 years old or showed evidence of impaired renal, cardiac, or hepatic function,

and all received 100,000 units of penicillin 4-hourly for 7 days. On the 2nd, 3rd, and 4th days caronamide was given 4-hourly, half an hour before meals. From 7 patients receiving 2 g., and from 5 receiving 4 g., 1 to 3 blood samples were taken 4 hours after administration of the drugs, and from 5 patients receiving 4 g. samples were taken after 2 hours. Serum was stored at —20° C. The penicillin content was estimated by a serial dilution method, and the caronamide by the colorimetric method of Ziegler and Sprague.

It was found that a concentration of 25 mg. of caronamide per 100 ml. of serum was necessary to achieve a twofold increase in penicillin concentration. A dose of 4 g. 4-hourly was required to produce a serum caronamide concentration of 30 mg. per 100 ml. 4 hours after administration. To obtain a fourfold or greater increase in penicillin content concentrations of caronamide exceeding this were required. The effect of the drug was cumulative, maximum penicillin levels being reached after 2 days. Nausea in 3 patients receiving 4 g. of caronamide was the only symptom of toxicity recorded.
J. E. M. Whitehead.

The Role of High Blood Penicillin Levels Achieved with Caronamide in Penetrating the Blood-Brain Barrier. JANOWITZ, H. D., SCHNEIERSON, S. S., SUSSMAN, M. L., and KING, F. H. (1948). *J. Lab. clin. Med.*, 33, 933.
In patients given caronamide in doses of 4 g. 3-hourly, injection of various doses of penicillin yielded high levels of penicillin in the blood, with concomitant high levels in the cerebrospinal fluid. In the absence of caronamide, the levels in blood and cerebrospinal fluid were far lower and far less persistent. It is suggested that caronamide might make intrathecal injection of penicillin unnecessary.
J. D. Judah.

BIOCHEMISTRY

Plasma Protein Studies on Normal Newborn and Premature Infants. I. Plasma Protein Values for Normal Full Term and Normal Premature Infants. II. Use of Concentrated Normal Human Serum Albumin in Treatment of Premature Infants. MCMURRAY, L., ROE, J. H., and SWEET, L. K. (1948). *Amer. J. Dis. Child.*, 75, 265.
The plasma albumin content was found to be higher in full-term than in premature infants. The mean value for albumin in full-term infants was 4.8 g. per 100 ml. (normal adult range, 4.5 to 6.5 g.) and in premature infants 4.3 g. per 100 ml. The globulin values for both groups were within the normal adult range, 1.2 to 2.2 g. per 100 ml.

The purpose of the second part of the study was to assay the value of concentrated normal human serum albumin in the treatment of premature infants. The serum used contained 25 g. of albumin per 100 ml. From birth up to 3 or 4 weeks 2 ml. of the concentrated serum albumin per lb. (454 g.) body weight was given intravenously once or more a week. Besides a significant rise in plasma albumin, the treated premature infants, compared with controls, gained in weight more rapidly, had more vigour, and were less prone to intercurrent illnesses. Further study of this form of treatment for premature infants is indicated. *W. G. Wyllie.*

The Copper and Iron Content of the Blood Serum in Normal Subjects. (Om Kobber- og Jernindholdet i Blodserum hos Normale.) BRENDSTRUP, P. (1948). *Ugesk. Loeg.*, 110, 945.
The iron and copper content of serum was estimated in 80 normal adults.

The values for iron in men varied from 80 to 177 μg. per 100 ml., with an average of 126 μg. per 120 ml., and in women from 54 to 163 μg. per 100 ml., with an average of 98 μg. per 100 ml. The values for copper in men varied from 85 to 162 μg. per 100 ml., with an average of 113 μg. per 100 ml., and in women from 89 to 152 μg. per 100 ml., with an average of 120 μg. per 100 ml. No correlation was found between copper and iron levels in any individual.

The Effect of Sodium Bicarbonate on the Renal Excretion of Salicylate. WILLIAMS, F., and LEONARDS, J. R. (1948). *J. Pharmacol.*, 93, 401.
In normal female dogs, an oral dose of 0.5 g. of sodium salicylate every 3 hours was well tolerated. Serum salicylate content rose gradually to a level of —about 60 mg. per 100 ml. after 2 days. When the blood level was stabilized, the dog received 1.6 g. of sodium citrate and 0.25 g. of sodium bicarbonate in addition to the regular dose of sodium salicylate. The serum salicylate level fell within the next 2 days to a final low value of 15 mg. per 100 ml. When the alkali administration was stopped the salicylate level began slowly to rise again. The urinary excretion of salicylate showed a significant increase with the administration of bicarbonate, and this increase was of a sufficient size to account for the decreased blood level.

The effect of alkali on the renal clearance of salicylate in 4 human subjects was similar to that observed in the dog. Sodium bicarbonate increased the salicylate clearance but the effect was not as striking as that in the dog, partly because there appeared to be more salicylate bound in the plasma protein in the human subject. The increase in clearance, as the pH of the urine increased, was due mainly to an increase in the excretion of unconjugated salicylate. *G. B. West.*

Heterophile Antibody Titer in Diseases Other than Infectious Mononucleosis. SCHULTZ, L. E. (1948). *Arch. intern. Med.*, 81, 328–333.
On the basis of 160 tests on 57 patients it is shown that positive Paul–Bunnell reactions may be given by the sera of persons not suffering from infectious mononucleosis. Positive results were obtained in all of 6 cases of Hodgkin's disease, in 1 of 3 cases of agranulocytosis, 2 of 2 cases of monocytic leukaemia, 1 of 3 cases of polycythaemia, 5 of 8 cases of sarcoma other than Hodgkin's disease, and 19 of 29 cases of tuberculosis. The "diagnostic level" is taken as a positive result in a dilution of 1 in 56 or more. The tests on individual patients show considerable variation from time to time.
C. L. Oakley.

The Sugar Content of Cerebrospinal Fluid in Tuberculous Meningitis and its Relation to the Reducing Properties of Streptomycin. (La glicorachia in corso di meningite tubercolare e suoi rapporti con il potere riducente della streptomicina). ALVARO, M., and NICOLA, M. (1948). *Riv. Clin. pediat.*, 46, 372–380.
The authors show by *in vitro* experiments that streptomycin itself has a reducing action in the Hagedorn–Jensen method of estimating sugar in C.S.F., that this action is most marked in the more dilute solutions of the drug, and that estimations of sugar in C.S.F. therefore give unreliable results in cases treated with streptomycin. *S. S. B. Gilder.*

HAEMATOLOGY

Vitamin B$_{12}$ Therapy in Pernicious Anemia. 1. Effect on Hematopoietic System : Preliminary Report. HALL, B. E., and CAMPBELL, D. C. (1948). *Proc. Mayo Clin.,* 23, 584.

Eleven patients with P.A. in relapse have been treated with Vit. B$_{12}$, with responses comparable with those obtained by liver. The bone marrow became normoblastic within 48–72 hours of the beginning of therapy. The doses given varied from 1 μg.–5 μg. daily or 25 μg. once a week. It is concluded that 1 μg. is equivalent in potency to about 1 U.S.P. unit of liver extract.

L. J. Davis.

Vitamin B$_{12}$ Therapy in Pernicious Anemia. II. Effect on the General Clinical and Neurologic Manifestations: Preliminary Report. HALL, B. E., and CAMPBELL, D.C. (1948). *Proc., Mayo Clin.,* 23, 591.

Oral and gastro-intestinal symptoms rapidly disappeared on treatment with Vit. B$_{12}$. Three patients with neurological complications showed remarkably good improvement. *L. J. Davis.*

Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia X. Activity of Vitamin B$_{12}$ as Food (Extrinsic) Factor. BERK, L., CASTLE, W. B., WELCH, A. D., HEINLE, R. W., ANKER, R., and EPSTEIN, M. (1948). *New Engl. J. Med.,* 239, 911.

Evidence is presented which indicates that Vit. B$_{12}$ when fed by mouth to patients with pernicious anaemia is more effective if neutralized normal gastric juice is fed at the same time. It is suggested that the extrinsic factor in food is identical or closely similar to Vit. B$_{12}$, and that the intrinsic factor in gastric juice is necessary for the proper assimilation of Vit. B$_{12}$ or related substances present in food. Reference is made to the presence in the faeces of a patient with untreated P.A. of relatively large amounts of Vit. B$_{12}$. This is presumed to be synthesized by the intestinal bacteria. *L. J. Davis.*

Hemolytic Anemia with Hemoglobinuria. STATS, D., WASSERMAN, L. R., and ROSENTHAL, N. *Amer. J. clin. Path.,* 18, 757.

The authors set out their experience of haemolytic anaemia accompanied by haemoglobinuria. The cases belonged to 4 types: the Marchiafava-Micheli syndrome, those associated with cold agglutinins, those due to drugs, and an idiopathic variety. They consider the methods of investigation and stress the value of spectroscopy and the significance of haemosiderinuria. They believe splenectomy to be contraindicated and that blood transfusions are the most valuable form of therapy.

Severe Haemolytic Anaemia with Formation of Heinz Inclusion Bodies in a Premature Infant. (Deletäre hämolytische Anämie mit " Spontan-Innenkörper "—Bildung bei Frühgeburt.) GASSER, C., and KARRER, J. (1948). *Helv. pediat. Acta,* 3, 387.

The case is recorded of a severe fatal haemolytic anaemia of unknown origin in a premature infant. Many of the red corpuscles contained inclusion bodies demonstrated by vital staining. They appeared to be similar in character to Heinz bodies which are known to be produced by poisons such as aniline and the sulphonamides, etc. *A. Piney.*

Studies on the Destruction of Red Blood Cells. IV. The Spleen as a Source of a Substance Causing Agglutination of the Red Blood Cells of Certain Patients with Acquired Hemolytic Jaundice by an Antihuman Serum Rabbit Serum (Coombs' Serum). WAGLEY, P. F., CHUSHEN, S., GARDNER, F. H., and CASTLE, W. B. (1948). *J. Lab. clin. Med.,* 33, 1197.

Saline extracts of spleens from patients with haemolytic anaemias and other disorders were tested for their power of sensitizing normal corpuscles to the agglutinating action of Coombs' serum. Positive results were obtained with four spleens from patients with acquired haemolytic anaemia. It was also observed that blood from the spleens of patients with acquired haemolytic anaemia was more strongly agglutinated than was peripheral blood. *J. Maclean Smith.*

Exchange Transfusion in Haemolytic Disease of the Newborn. MOLLISON, P. L. and CUTBUSH, M. (1948). *Lancet,* 2, 522.

Thirty out of a series of 63 babies with haemolytic disease of the newborn were treated by exchange transfusion. Only seven infants died after transfusion. This procedure is advocated when the cord haemoglobin level is less than 14.5 g. or when more than 4 mg. of bilirubin is present. The presence in the stained blood film of 10 or more nucleated red cells per 100 leukocytes is an additional indication.

Relapse of Pernicious Anaemia During Maintenance Therapy with Folic Acid. MOLLIN, D. L. (1948). *Lancet,* 2, 928.

The case history is described of a man with typical pernicious anaemia who was treated with folic acid. After a fairly satisfactory initial response he gradually became refractory to maintenance therapy and eventually his blood count continued to fall even when given 20 mg. folic acid daily. At this point he was given liver injections and rapidly responded.

Geoffrey McComas.

Amount of Prothrombin Used up During Coagulation of Venous and Capillary Blood. A New Method of Investigation of Haemorrhagic Syndromes. (La consommation de la prothrombine pendant la coagulation du sang veineux et du sang capillaire. Nouvelle méthode d'investigation des syndromes hémorragiques.) SOULIER, J. P. (1948). *Rev. Hémat.,* 3, 302

This method measures the amount of prothrombin not converted into thrombin during the coagulation of blood. Normally this only represents 10–20% of the total prothrombin, but in haemophilia a much higher proportion is left unconverted.

The test (time of the clotting of a standard fibrinogen solution by the patient's serum in the presence of excess thromboplastin) is a more delicate indicator of a coagulation defect than the whole blood coagulation time and is useful in the diagnosis of haemophilia.

By performing the test on samples of serum obtained from venous and capillary blood (the latter contaminated with tissue thromboplastin) the effect of thrombocytopenia can be demonstrated. In this case the prothrombin content in the venous sample is considerably higher than in the capillary sample.

The Value and the Limitations of the Coagulation Time in the Study of the Hemorrhagic Diseases. QUICK, A. J., HONORATO, R., and STEFANINI, M. (1948). *Blood*, 3, 1120.

The authors describe the Lee and White method of measuring the rate of coagulation *in vitro* and discuss the various factors which will influence the results. A temperature of 37° C. is recommended.

Only in haemophilia are coagulation times regularly increased, although even here a normal rate of coagulation may sometimes be met with. In other haemorrhagic disorders the coagulation times are usually normal (except in the rare condition afibrinogenaemia).
R. Winston Evans.

Studies of Hemophilia. I. The Control of Hemophilia by Repeated Infusions of Normal Human Plasma. ALEX-ANDER, B., and LANDWEHR, G. (1948). *J. Amer. med. Ass.*, 138, 174.

The authors describe how four patients have been treated by intravenous infusions of 100–180 ml. human plasma given 3–4 times a week. The beneficial effect on the coagulation time lasted for 24–72 hours. No resistance to transfusion developed and the clinical results were satisfactory. *Alexander Brown.*

Hemorrhagic Diathesis Associated with the Presence of an Anticoagulant in Circulating Blood. Case Report and Laboratory Studies. SOULIER, J. P., and BURSTEIN, M. (1948). *Blood*, 3, 1188.

The rare incidence is described of a haemophilia-like syndrome due apparently to the presence of a circulating anticoagulant. The patient's blood was found to have a pronounced anticoagulant effect on normal blood. Its own prolonged coagulation time could not be restored to normal by the addition of normal plasma as in haemophilia. *Marjorie Le Vay.*

Circulating Anticoagulant as a Cause of Hemorrhagic Diathesis in Man. CONLEY, C. L., RATHBUN, H. K., MORSE, W. I., ROBINSON, J. E. (1948). *Bull. Johns Hopk. Hosp.*, 83, 288.

The authors report three cases in which haemorrhagic diatheses appeared to be due to the presence of circulating anticoagulants. The anticoagulants appeared to inhibit an early phase in the coagulation process.

Thrombopenic Purpura : the Failure of Direct Blood Transfusion to Raise the Platelet Level. LAWRENCE, J. S., VALENTINE, W. N., and ADAMS, W. S. (1948). *J. Lab. clin. Med.*, 33, 1077.

In previous experiments the authors have demonstrated that the platelet count in cats may be raised for a few days following "direct" blood transfusions. In man, however, the low platelet counts of two patients suffering from thrombocytopenic purpura were not raised when they were transfused by a similar technique.
Douglas H. Collins.

A Study of the Bone Marrow from Thirty-six Patients with Idiopathic Hemorrhagic (Thrombopenic) Purpura. DIGGS, L. W., and HEWLETT, J. S. (1948). *Blood*, 3, 1090.

No differences were found between the total megakaryocyte counts in marrow obtained by sternal puncture between the 36 patients with idiopathic thrombocytopenic purpura and 50 control patients suffering from various non-haemorrhagic disorders, and no constant relation was found between the megakaryocyte count in the cases of purpura and prognosis and response to splenectomy. However, differential counts showed that in purpura there was an unusual proportion of primitive megakaryocytes not engaged in forming platelets. This relative immaturity of the megakaryocytes was not significantly affected by splenectomy.
M. C. G. Israëls.

The Bone Marrow on Sternal Aspiration in Multiple Myeloma. BAYRD, E. D. (1948). *Blood*, 3, 987.

A review of 51 cases examined by sternal puncture at the Mayo Clinic. The tumour plasma cells are believed to be derived from reticulum cells. Those cases in which the cells were most immature and variable in type terminated the quickest (mean survival 6.3 months). In contrast, seven patients in whom the plasma cells were of a mature uniform type survived 2–7 years. *J. Maclean Smith.*

Paroxysmal Cold Hemaglobinurias. BECKER, R. M. *Arch. intern. Med.*, 81, 630.

In this paper are reviewed 37 cases of syphilitic paroxysmal haemoglobinuria. The characteristic features of this disease are contrasted with those of haemoglobinuria associated with cold agglutinins.

Are Non-nucleated Erythrocytes Formed by Budding off of Cytoplasm from Normoblasts ? (In English.) BOSTRÖM, L. (1948). *Acta med. scand.*, 131, 303.

In this paper evidence is produced which is held to support the interesting but heterodox hypothesis that several erythrocytes may be derived in the bone marrow by budding off from the cytoplasm of a single erythroblast. *A. K. Powell.*

Serological Examination of the Placenta. (Serologisch placentaonderzoek.) VAN BOLHUIS, J. H. (1948). *Ned. Tijdschr. Geneesk.*, 92, 2950.

The author claims that the development of the antibodies in pregnancy is due to antigens in the placenta of the infant rather than in his corpuscles. Practically all the placentae of affected infants were found to have the power of absorbing Rh antibodies, but only about 10% of the placentae of unaffected Rh positive infants had this property. If it is assumed that about one-half of the mothers are capable of forming antibodies (as is suggested by the results of transfusion of Rh+ blood into Rh negative recipients), the 5% incidence of antibodies in Rh negative women bearing Rh positive children can be explained. *J. J. van Loghem.*

Adult Gaucher's Disease, with Special Reference to the Variations in its Clinical Course and the Value of Sternal Puncture as an Aid to its Diagnosis. GROEN, J., and GARRER, A. H. *Blood*, 3, 1221.

The authors give the histories of nine personally studied cases of Gaucher's disease. They stress the frequency of bone pains due to skeletal involvement and the value of sternal puncture as an aid to early diagnosis. Cell types intermediate between reticulum cells and Gaucher cells may be found. Splenectomy does not alter the rate of progress of the disease.

The Bone Marrow as a Diagnostic Aid in Acute Disseminated Lupus Erythematosus. Report on the Hargraves' "L. E." Cell. HASERICK, J. R., and SUNDBERG, R. D. (1948). *J. invest. Derm.*, 11, 209.

The "L. E." cell of the bone marrow is a polymorph leucocyte which has engulfed a round homogeneous

Feulgen-positive mass. These cells were present in the bone marrow of four out of five patients with acute disseminated lupus erythematosus. *E. Lipman Cohen.*

Influence of ABO Incompatibility on Rh Antagonism. (L'influence de l'incompatibilité du système ABO sur l'antagonisme Rh.) LOGHEM, J. J. VAN, and SPAANDER, J. *Rev. Hémat.*, 3, 276.

Evidence is presented suggesting that ABO incompatibility may cause the death of the foetus at an early age.

A Serum which Demonstrates the Co-dominance of the Blood-group Gene O with A and B. BOORMAN, K. E., DODD, B. E., and GILBEY, B. E. (1948). *Ann. Eugen., Camb.*, 14, 201.

A serum agglutinating all bloods containing O and A_2 is described. It agglutinated OO, OA_2, and A_2A_2 cells more strongly than A_1O, A_1A_2, A_2B, or BO cells. The authors believe their serum to be a pure anti-O serum and that bloods of Group O, A_2, and A, respectively are really OO, AO, and AA_1 in type. The fact that anti-A sera contain anti-A as well as anti-A_1 fits in with this hypothesis. *John Murray.*

The Rh Antigen D^u. RACE, R. R., SANGER, R., and LAWLER, S. D. (1948). *Ann. Eugen., Camb.*, 14, 171.

The D^u genes, of which 12 different types have been recognized, are thought to have arisen as a series of graduated mutations from the D gene. Cells containing the D^u gene are agglutinated by some anti-D sera, but not by all. They are, however, sensitized to the Coombs' test by the great majority of incomplete anti-D sera. The picture is a complicated one. *John Murray.*

A Simple and Rapid Method for Demonstrating Sickling of the Red Blood Cells : The Use of Reducing Agents. DALAND, G. A., and CASTLE, W. B. (1948). *J. Lab. clin. Med.*, 33, 1082.

Sickling of certain red blood cells occurs when oxyhaemoglobin is changed to reduced haemoglobin. A mixture of ascorbic acid and sodium bisulphite or 2% sodium bisulphite alone may be used as reducing agents and sickling observed within 15–60 minutes when these reagents are added to susceptible blood. Zenker's fluid causes reversal to a discoidal form, but formalin preserves the sickled shape and is thus preferable for fixing post-mortem tissues. *Douglas H. Collins.*

MORBID ANATOMY AND HISTOLOGY

Transitional Endometrium. (In English.) ORAM, V. (1948) *Acta Obstet. gynec. scand.*, 28, 188.

Transitional endometrium is that normally found in the late interval phase, that is, the phase between proliferation and secretion. The typical picture of this phase is found with striking frequency in routine histological examinations. The characteristic picture is as follows. The amount obtained by curettage is copious and the mucous membrane thick and almost hyperplastic. The stroma is loose and oedematous and highly vascularized. The epithelial cells are cylindrical with a central nucleus. The cells are vacuolated and the vacuoles contain glycogen. The lumen of the gland does not contain secretion. The appearance suggests that development of the endometrium has suddenly ceased. The picture is in its own way as characteristic as that of cystic hyperplasia. A

study of the literature shows that these findings have been discussed several times but have never been systematically examined.

An analysis of 224 cases in which the typical picture was found is given. In many patients bleeding occurs from this type of transitional endometrium. Hormonal treatment was tried in some cases, but a normal secretory phase was rarely re-established. A few hormone analyses were made and these showed excess of gonadotropic hormone but decreased output of oestrogenic hormone. Further and more extended hormone analysis is definitely required. Hormone imbalance is probably the basis of the condition but it is as yet impossible to say whether the imbalance arises primarily in the ovaries, the pituitary, or elsewhere. *Josephine Barnes.*

Melanomas of Childhood. SPITZ, S. (1948). *Amer. J. Path.*, 24, 591.

Thirteen cases of melanoma, occurring in children between the ages of 18 months and 12 years and therefore called juvenile melanoma, were studied. Comparison was made with 50 cases of benign naevus of childhood, and with 17 melanomata occurring between the ages of 14 and 19 years. Histologically, in contrast to the benign naevi, the juvenile melanomata were pleomorphic. Half of them contained prominent giant cells, but except for this were histologically malignant. All but 1 patient, however, remained alive and well for periods of up to 13 years after simple excision. The adult-type melanomata showed gross histological variation, but only 1 contained giant cells. Of these patients 71% were dead within 18 months. It is stated that substantiated cases of malignant behaviour of melanomata in childhood are few. There is a marked rise in the degree of malignancy after puberty although the tumours are histologically similar to those behaving benignly. A hormonal influence is postulated to account for this difference. *Charles Pike.*

Histiocytic Reticulosis in Infants. REYE, R. D. K. (1948). *Med. J. Aust.*, 2, 509.

The author presents cases illustrating four diseases: 1) Letterer–Siwe disease; (2) eosinophilic granuloma of bone; (3) infective reticulo-endotheliosis; (4) Hand–Schüller–Christian disease.

The examples described illustrate the difficulty of separating these conditions, and the author suggests a unifying classification as follows:

Histiocytic reticulosis (inflammatory): (1) Acute—Letterer–Siwe's disease, and rapidly progressive instances of eosinophilic granuloma of bone. (2) Subacute—Eosinophilic granuloma of bone, with or without visceral lesions. (3) Chronic—slowly progressing histiocytic or lipogranulomatous lesions of bone or viscera, and certain fibrous lesions. The majority of cases of Hand–Schüller–Christian disease would fall into this group.

Sarcoma Arising in Irradiated Bone. Report of Eleven Cases. CAHAN, W. G., WOODARD, H. Q., HIGINBOTHAM, N. L., STEWART, F. W., and COLEY, B. L. (1948). *Cancer*, 1, 2.

The literature on development of sarcoma in irradiated bone is reviewed, the experimental production of bone sarcoma by irradiation in animals and the reported cases developing in human subjects being discussed. Eleven further cases are presented in detail ; osteogenic sarcoma developed in irradiated bones 6 to 22 years after treatment of benign bone lesions or in normal bone after irradiation of another lesion. The average age was 34 years. The histologically confirmed cases included 2

giant-cell tumours, one bone cyst, and one ossifying fibroma ; there was one case of sarcoma in the second rib following post-operative irradiation of the chest wall after mastectomy. Ten cases were treated by x-rays and one case by radon pack. Estimated tumour doses were generally over 3,000 r, though one bone cyst had received only 1,550 r. The most constant clinical sign of malignancy was the sudden onset of progressive pain ; this sign, in an area treated by radiation 5 years or more previously, calls for immediate biopsy examination. The authors believe that recognition of this possibility will lead to discovery of more cases ; they are consequently opposed to x-ray therapy for non-malignant tumours of bone, including giant-cell tumours. *J. Walter.*

Bone Lesions in Eosinophilic Granuloma, Hand–Schüller–Christian Disease, and Letterer–Siwe Disease. PONSETI, I. (1948). *J. Bone Jt Surg.*, 30A, 811.

This is an account of 8 cases, varying from single eosinophil granuloma to well-marked Hand–Schüller–Christian disease, to illustrate the author's contention that these diseases are varying responses to a common unknown aetiological factor.

Transition of Boeck's Sarcoidosis to Miliary Tuberculosis. (Ubergang von Boeck'scher Krankheit in Miliartuberkulose. Ein Beitrag zum Problem der 'atypischen' Tuberkulosen). FREY, U. (1948). *Helv. med. Acta.*, 15, 129.

The author reports the case of a man, aged 29, who suffered from Boeck's disease affecting the hilar, cervical, and axillary lymph nodes for 4 years before death. The diagnosis of Boeck's disease was confirmed by lymph node biopsy examination. The disease took a benign course until suddenly, 4 years after it had been diagnosed, the patient developed a fatal tuberculous meningitis. Necropsy and histological examination revealed in several organs old and largely fibrotic granulomata like those in Boeck's disease. In addition, fresh typical miliary tubercles and changes representing a transition from the lesions of Boeck's disease to those of tuberculosis were found. On account of the histological findings and the clinical history the author considers this case to be one of transition of Boeck's disease into miliary tuberculosis.

Boeck's disease spreads by haematogenous dissemination and appears very similar to "chronic miliary tuberculosis." The case described is thought to support the theory of a tuberculous aetiology of Boeck's disease and the author considers the latter to be a special form of tuberculosis. He therefore proposes to call it "atypical tuberculosis, type Boeck." The prognosis of "atypical tuberculosis" is not always favourable, because of the possible transition into miliary tuberculosis.
R. Schade.

Histologic Features of Carcinoma of the Cardio-esophageal Junction and Cardia. MCPEAK, E., and WARREN, S. (1948). *Amer. J. Path.*, 24, 971.

The histological structure of 65 carcinomata of the oesophago-gastric junction is reviewed. The authors conclude that the adeno-acanthomata and some papillary adenocarcinomata of this region may arise from the oesophageal glands, that other papillary adenocarcinomata arise from the gastric glands, and that in general adenocarcinomata of this region are better differentiated and less productive of metastases than those of other parts of the stomach. *R. A. Willis.*

Cytological Studies of Sputum and Bronchial Secretions in the Diagnosis of Cancer of the Lung. LIEBOW, A. A., LINDSKOG, G. E., and BLOOMER, W. E. (1948). *Cancer*, 1, 223.

The Diagnosis of Bronchogenic Carcinoma by Smears of Bronchoscopic Aspirations. MCKAY, D. G., WARE, P. F., ATWOOD, D. A., and HARKEN, D. E. (1948). *Cancer*, 1, 208.

In these two papers the application of the smear-technique is discussed, and two large series of cases are analysed. Those interested should consult the original papers.

The Pathogenesis of Splenomegaly in Hypertension of the Portal Circulation; "Congestive Splenomegaly." MOSCHCOWITZ, E. (1948). *Medicine, Baltimore*, 27, 187.

This is a discussion of the pathology of the spleen in 86 cases, in which the common factor is resistance or obstruction to the venous return. The cases are divided into three main groups: (a) where the obstruction is of post-hepatic origin, such as constrictive pericarditis and mitral stenosis; (b) hepatic cirrhosis of various types; and (c) pre-hepatic obstruction due to thrombosis of the portal or splenic vein, or of both. The differences in the spleen in the various groups are regarded as degrees of the same process, depending, first, on the duration of the hypertension, and, secondly, on the proximity to the spleen of the obstructing lesion; for instance, the changes are minor in constrictive pericarditis, but pronounced in the presence of an organized and recanalized thrombus in the splenic vein. As would be expected, a well-developed collateral circulation is present only when the hypertension is of long standing, and further lesions, such as siderotic nodules and myeloid metaplasia, are found only in similar circumstances. *W. S. Killpack.*

Relation between Structural and Functional Alterations of the Liver. FRANKLIN, M., POPPER, H., STEIGMANN, F., and KOZOLL, D. D. (1948). *J. Lab. clin. Med.*, 33, 435.

The authors carried out 165 liver biopsies on 130 patients suffering from various types of liver disease. The main histological features were statistically compared with the results of a series of liver function tests carried out within 2 days of the biopsy. Among the many results the following may be quoted. Diffuse liver-cell damage was found to be significantly correlated with cephalin–cholesterol flocculation, thymol turbidity, albumin–globulin ratio, and bromsulphalein retention, but not with total serum protein or with alkaline phosphatase. Focal necrosis and fatty change were not associated with any significant change in the liver function tests. Regeneration was correlated with increased thymol turbidity. (The original should be consulted for other results.) The authors are careful to point out that the statistical correlations disclosed do not prove that an abnormal function is necessarily caused by the associated pathological change. *Douglas H. Collins.*

Cicatrizing Enteritis (Regional Ileitis) as a Pathologic Entity. Analysis of One Hundred and Twenty Cases. WARREN, S., and SOMMERS, S. C. (1948). *Amer. J. Path.*, 24, 475.

This paper presents the results of analysis of 120 cases of regional ileitis, with a review of the literature. Empha-

sis is laid on macroscopical and microscopical morbid anatomical features, descriptions being given of acute, subacute, and chronic phases. The chronic phase—the most common—is dealt with in detail. Early changes, not previously described, were observed in the gut and lymph nodes. In the gut, between the lymph nodes and muscularis mucosae, there was observed focal proliferation of lymphatic endothelium, leading to obstruction and dilatation and oedema. Similar changes are seen later in the submucosa and subserosa; the endothelial cells coalesce to form giant cells; eosinophils and lymphocytes surround the endothelial masses. Similar changes occur in mesenteric lymph nodes and vessels. Ulceration does not appear until oedema is considerable; the resulting non-specific inflammatory changes often overshadow the early granulomata. Differential diagnosis is seldom a problem but may be difficult. The aetiology remains unknown. As regards pathogenesis, lymphatic blockade is considered of fundamental significance.

R. R. Wilson.

The Pathology of Infectious Mononucleosis. Custer, R. P., and Smith, E. B. (1948). *Blood*, 3, 830.

This account of the morbid histology of infectious mononucleosis, based on 9 necropsies and numerous biopsies, describes the characteristic hyperplasia of the lymphoid tissues. The spleen, which was invariably enlarged at the height of the disease, was packed with lymphoid cells. Splenic follicles and trabeculae were rendered less prominent than usual, and spontaneous rupture had occurred in 4 cases. The bone-marrow contained no abnormal cellular infiltration. Small aggregations of lymphocytes were found in the myocardium. In one case a pneumonic exudate in the lungs consisted of lymphoid cells. Periportal infiltrations of lymphocytes were sometimes excessive in the liver. In 4 out of 6 brains examined a mild or moderate meningo-encephalitis was observed, and in 2 cases there was a distinct peripheral neuritis. *Douglas H. Collins.*

The Collection of Radioactive Iodine by the Human Fetal Thyroid. Chapman, E. M., Corner, G. W., Robinson, and Evans, R. D. (1948). *J. clin. Endocrinol.*, 8, 717.

The series investigated suggests that thyroid function is initiated in the foetus at 14.5 weeks.

Congenital Cystic Disease of the Lung. Bowden, K. M. (1948). *Med. J. Aust.*, 2, 311.

The author has studied 20 new cases of congenital cystic disease of the lung, and summarizes his findings as follows. Congenital cysts of the lung occur either as solitary large cysts or as multitudinous, smaller cysts. They have been found in foetuses, and at all ages. In most cases they contain air, though occasionally they are filled with a gelatinous substance. Histological examination of a typical case will reveal an epithelial lining of columnar cells, bordered by a basement membrane, while the underlying tissues consist of fibrous and smooth muscle tissue and elastic fibres. Cartilage and mucous glands may be present. Noteworthy features are the frequent absence of alveoli in the immediate neighbourhood, and the absence of carbon deposits, those parts of the lungs appearing pink and fleshy. The author interprets his findings as indicating an arrest of normal development of the bronchi. Other co-existent congenital abnormalities lend support to this assumption.

R. Salm.

Streptomycin in Tuberculous Meningitis in Childhood: The Pathological Findings in Six Fatal Cases. Montgomery, G. L. (1948). *Glasg. med. J.*, 29, 235.

The post-mortem appearances in 6 cases of tuberculous meningitis treated with streptomycin are described. In 4 the primary focus was still alive. In one activity was arrested, while in another the focus was not found and may have regressed. In all cases there were visceral tubercles which had undergone hyaline change, some being completely hyalinized. Other tubercles showed activity, but there were no histiocytic abscesses as described by Wright. Death in spite of treatment was due to a continuation of the meningitic process. Attention is drawn to the hyalinization of splenic arterioles (a condition normal in older subjects but not in childhood).

D. M. Pryce.

Histopathology of Virus Encephalomyelitis. Scheinker, I. M. (1948). *Arch. Path.*, 45, 289.

The histological lesions in 3 forms of encephalomyelitis, the epidemic encephalitis of von Economo, Japanese (Type B) epidemic encephalomyelitis, and anterior poliomyelitis, are compared. In these three conditions the nerve cells of the grey matter are primarily attacked and the white matter is left relatively intact. The differences in distribution are described in detail.

Tertiary Syphilis of the Uterine Body and Adnexa. (Sifilis terciaria do corpo e anexos uterinos.) De Souza Rudge, W., and Delascio, D. (1948). *Obstet. Ginec. lat. amer.*, 6, 393.

A 23-year-old married woman complained of vague abdominal pain of 3 months' duration. On examination, her uterus was found to be enlarged to the size of a foetal head at term, mobile, and irregular. At laparotomy, the presence of multiple adhesions suggested that a uterine fibroid was undergoing malignant transformation. Subtotal hysterectomy, bilateral salpingectomy, and left ovariectomy were carried out. Post-operative progress was uneventful. Histological examination revealed numerous gummata in the myometrium and the ovary. The Wassermann reaction was strongly positive. This is stated to be the fourth authentic case published of tertiary syphilis of the uterus. *S. S. B. Gilder.*

Muscle Histology in Rheumatic and Control Cases: A Study of One Hundred and Nineteen Biopsy Specimens. Desmarais, M. H. L., Gibson, H. J., and Kersley, G. D. (1948). *Ann. rheum. Dis.*, 7, 132.

Biopsies of skeletal muscle from 56 cases of rheumatoid arthritis have shown focal round-celled (mainly lymphocytic) infiltration in the endomysium and perivascular tissues in 60.7% of cases. Similar changes were found in 1 of 3 examples of Still's disease. Negative results were obtained in a variety of other forms of arthritis.

Chorioangiofibroma. Horn, H. W. (1948). *Med. J. Aust.*, 2, 183.

The paper records an unusually large angioma of the placenta, 8 cm. in main diameter, the edge of which presented along with the child's head and caused serious haemorrhage during the second and third stages of labour. The five photomicrographs show clearly the capillary angiomatous structure of the tumour, to which the author applies the name "chorioangiofibroma" used by Siddall in his review of 130 reported tumours of this kind (*Amer. J. Obstet. Gynec.*, 1924, 8, 554).

R. A. Willis.

THE LABORATORY DIAGNOSIS OF LYMPHOGRANULOMA VENEREUM

BY

S. P. BEDSON, C. F. BARWELL, E. J. KING, AND L. W. J. BISHOP

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Although the literature on this subject is already quite considerable there is still some doubt as to the exact value of the different laboratory procedures available for the diagnosis of lymphogranuloma venereum. This does not, of course, apply to the demonstration of lymphogranuloma venereum virus in material from a suspected case of this disease, for this, as in any infective process, is of unequivocal value. Unfortunately the isolation and identification of lymphogranuloma venereum virus, though always worth undertaking, particularly in the early stages of the disease, is time-consuming and, in our limited experience, not nearly so readily achieved as some other workers have found (Wall, 1946). In the work here reported we have been more concerned with those laboratory tests, the Frei test and the lymphogranuloma venereum complement fixation test, which are of easy application and give an answer in a relatively short time. Much of the earlier work with these tests was done before Rake and his colleagues (1941) had drawn attention to the close relationship which exists between the viruses of the psittacosis-lymphogranuloma group. The extensive sharing of antigens, which is one of the important features of this relationship, is responsible for a high degree of cross reaction in complement fixation tests made with these viruses and their antisera (Rake, Eaton, and Shaffer, 1941) reducing them to a group specificity. The consequences for the Frei test are similar ; a positive reaction to this test as at present practised is not confined to lymphogranuloma venereum but can also occur in infections with viruses of the psittacosis group (Rake, Eaton, and Shaffer, 1941). And although some of the more recent work on the Frei test and the complement fixation test in lymphogranuloma venereum has been done with Rake's important findings in mind the precise value of these two tests in the diagnosis of lymphogranuloma venereum is still far from clear. It was in an endeavour to remedy this deficiency and to find out if it would be possible to devise intra-

dermal and serological tests of greater specificity that the following investigations were made.

The Complement Fixation Test

Technique.—The antigen was prepared from the yolk sacs of eggs inoculated on the fifth day of incubation and taken down four to five days later. Yolk sacs were ground in Tenbroek tubes and suspended in phosphate buffer pH 7.6, 2 ml. per yolk sac. After sedimentation in the refrigerator or a preliminary centrifugation to get rid of gross particles and of yolk, the partially clarified suspension was centrifuged for one and a half to two hours on an angle centrifuge at 5,000 revolutions per minute, the supernatant fluid discarded, and the deposit suspended to half volume in saline. A further period of sedimentation in the refrigerator for 24 to 48 hours resulted in the deposition of more extraneous material, which was discarded. If smears of this final suspension stained by Castaneda's method showed a satisfactory virus content, the suspension was steamed for 20 minutes, sodium azide added in a final concentration of 0.3% as preservative, and the antigen titrated with known lymphogranuloma venereum positive and negative sera. Such an antigen has rarely been found to be anticomplementary and it keeps its activity unchanged for at least several months. The use of phenol for enhancing antigenic activity, advocated by Nigg, Hilleman, and Bowser (1946), has not, in our hands, provided a better antigen than one prepared by the above method. One or two attempts to produce an antigen by the method of Smadel, Wertman, and Reagan (1943) were not sufficiently encouraging to induce us to adopt this more laborious method. The addition of formalin in 0.3% concentration in place of heat inactivation invariably made the antigen anticomplementary. A control antigen was prepared in a similar manner from normal yolk sacs of appropriate age.

Complement.—A satisfactory batch of guinea-pig serum was divided into quantities suitable for a day's test and stored in the dry ice-box. Retitration on the day of each test showed little or no change over 12 weeks. A dose of 2 M.H.D. was used.

The Test.—Falling doubling dilutions from 1 in 4 to 1 in 256 were prepared from the patients' sera which had been inactivated at 56° C. for 30 minutes.

Patients' serum, complement (2 M.H.D.), and antigen, 0.1 ml. of each, were added to tubes in this order and the volume in each tube made up to 0.8 ml. with saline. Each serum in highest concentration was put up with the control antigen and also without antigen to detect any anticomplementary activity; other controls consisted of known positive and negative sera. Fixation was for 30 minutes at room temperature followed by 30 minutes in the 37° C. water-bath. Sheep red cells

with this disease are first seen in the secondary stage when the development of an inguinal adenitis has drawn attention to the presence of infection, and by that time antibody formation is usually well advanced. Other patients, and this is particularly true of women, only become aware of infection when the disease has reached the tertiary stage and some lesion, most usually in

TABLE I
CORRELATION BETWEEN CLINICAL DIAGNOSIS AND LYMPHOGRANULOMA VENEREUM COMPLEMENT FIXATION TEST

Clinical Diagnosis of Lymphogranuloma Venereum	Total Number of Patients	Number of Cases giving Fixation with a Lymphogranuloma Venereum Antigen at Dilutions of								Positive Complement Fixation Test at 1/32 or over (%)
		<1/4	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
Confident... ...	32	0	0	0	4	7	11	10	0	87.5
Possible	31	7	1	6	10	2	3	2	0	22.6
Doubtful	67	57	3	2	4	1	0	0	0	1.5

in 5% suspension sensitized with 5 M.H.D. amboceptor were then added to each tube in 0.2 ml. quantities and the tubes returned to the 37° C. water-bath for 30 minutes when the first reading was taken. A second reading was made after the tubes had stood at room temperature over night. The least dilution of serum giving complete fixation of 2 M.H.D. complement in the presence of antigen (no haemolysis at either the first or second reading) was taken as the titre.

the rectum, draws attention to its presence. Only rarely is the disease seen early enough for the formation of antibodies to be followed, and reliance, therefore, has to be placed on a single observation. Sera from 130 patients were examined, the patients being divided into three groups according to whether the clinical diagnosis of lymphogranuloma venereum was confident, pos-

TABLE II
COMPARISON OF RESULTS OF COMPLEMENT FIXATION TESTS ON SERA FROM PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM WITH PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM HEATED (100° C.) ANTIGENS

Clinical Diagnosis	Number of Sera Tested	Number of Sera Giving		
		Same Titre with Homologous and Heterologous Antigens	Higher Titre with Homologous Antigen	Higher Titre with Heterologous Antigen
Lymphogranuloma venereum	19	16	3	0
Psittacosis	13	5	8	0

Diagnostic Value of a Single Complement Fixation Test.—An attempt was made first of all to determine the value of a single complement fixation test, and to this end the results of this test made on a series of patients suspected of having lymphogranuloma venereum were compared with the clinical findings. When using the presence of antibody in a patient's serum as evidence of active infection the ideal undoubtedly is to demonstrate a significant rise in antibody titre, but this can only be done if the case is seen early enough and this rarely happens in lymphogranuloma venereum. The majority of patients

sible, or doubtful. The results are recorded in Table I.

It will be seen that the serological findings show quite a close correlation with clinical diagnosis, and that in the " confident " group the majority of sera give titres of 1 in 32 or over, whereas in the " doubtful " group only one out of 67 sera comes in this high titre range and the majority had titres of less than 1 in 4. In the clinically possible group the titre scatter, as might be expected, was much greater. It seems justifiable to conclude from these findings that in a patient with symptoms compatible with a diagnosis of lymphogranuloma

venereum, a titre of 1 in 32 or over indicates active infection with lymphogranuloma venereum virus and that a titre of 1 in 16 is suggestive of this. Previous workers have reached much the same conclusion (Landau, 1946 ; Dulaney and Packer, 1947).

Attempts to Devise a More Specific Test.—In the complement fixation test as described above the effective antigen is the heat-stable one which is common to the viruses of the lymphogranuloma venereum–psittacosis group, and this means that the test would be positive in infections due to other members of the group. It is well recognized that this is so, and, as others have observed and we have amply confirmed in the course of this work, an antigen prepared from psittacosis virus in a similar manner could equally well be employed in the lymphogranuloma venereum complement fixation test. Smadel, Wertman, and Reagan (1943) state that, although this is so, sera from human cases of psittacosis give rather better fixation with the homologous antigen. This we can confirm. In Table II the results of complement fixation tests made with sera from cases of

TABLE III

TITRATION OF LYMPHOGRANULOMA VENEREUM AND PSITTACOSIS SERA WITH LYMPHOGRANULOMA VENEREUM AND PSITTACOSIS VIRUS HEATED AND UNHEATED

Serum		Lymphogranuloma Venereum Virus		Psittacosis Virus		Control Antigen	Saline
Clinical Condition	Dilution	Unheated	Heated (100° C.)	Unheated	Heated (100° C.)		
Lymphogranuloma venereum " G "	1/8	++++ ++++	++++ ++++	++++ ++++	++++ ++++	− −	− −
	1/16	++++ ++++	++++ ++++	++++ ++++	++++ ++++		
	1/32	++++ ++++	++++ ++++	++++ ++++	++++ ++++		
	1/64	++++ +++	++++ +++	++++ +++	++++ +++		
Psittacosis " Jo " ...	1/4	++++ ++++	++++ ++++	++++ ++++	++++ ++++	− −	− −
	1/8	++++ +++	++++ ++++	++++ ++++	++++ ++++		
	1/16	+++ ++	++++ ++++	++++ ++++	++++ ++++		
	1/32	± +	+++ +++	++++ +++	++++ +++		
Psittacosis " L " ...	1/8	++ +	++++ ++++	++++ ++++	++++ ++++	− −	− −
	1/16	− −	++++ ++++	++++ ++++	++++ ++++		
	1/32	− −	++++ ++++	++++ ++++	++++ ++++		
	1/64	− −	++++ +++	+++ +++	++++ +++		
Normal	1/4	− −	− −	− −	−	− −	− −
Saline		− −	− −	− −	− −	− −	− −

lymphogranuloma venereum and psittacosis and the two heated antigens used in parallel show that sera from human cases of psittacosis do tend to give a higher titre with the steamed psittacosis virus than with the heterologous antigen, though why this should be is difficult to understand. However, the difference is neither great enough nor sufficiently constant to make it of use diagnostically.

It is known that psittacosis virus contains a heat-labile antigen in addition to the stable group specific one (Bedson, 1936 ; Barwell, 1948), and there is evidence that the viruses of the psittacosis–lymphogranuloma venereum group each possess a specific antigen (Hilleman and Gordon, 1944 ; Hilleman, 1945 ; St. John and Gordon, 1947) which can be detected by neutralization tests made with antisera produced in the domestic fowl. Neutralization tests made with human and mammalian sera give no such clear-cut specificity. Presumably the specific antigen is the labile component, and the superiority of the fowl sera in neutralizing is due to the better response in the bird to this

antigen than occurs in man and mammals. Rake and Jones (1944) have also shown that these viruses, when grown in the yolk sac, produce species-specific labile toxins which are lethal to the mouse on intravenous inoculation, but the relationship of this work to that of Hilleman and his colleagues is not clear. However, there is evidence that mammalian anti-psittacosis sera do contain antibody to the labile antigen (Bedson, 1936), and it was thought that complement fixation tests made with fresh unheated lymphogranuloma venereum virus might show greater specificity. Accordingly, sera from cases of lymphogranuloma venereum and human psittacosis were titrated against suspensions of both viruses unheated and heated at 100° C. The results were disappointing. The majority of lymphogranuloma venereum sera showed little or no difference in titre with all four antigens, though the psittacosis sera usually gave a lower titre—sometimes remarkably so—with the unheated heterologous antigen. An extreme example of this is shown in Table III.

TABLE IV

ABSORPTION OF A LYMPHOGRANULOMA VENEREUM SERUM WITH STEAMED PSITTACOSIS VIRUS

Serum		Lymphogranuloma Venereum Virus		Psittacosis Virus		Control Antigen	Saline
Treatment	Dilution	Unheated	Heated (100° C.)	Unheated	Heated (100° C.)		
Serum W (lymphogranuloma venereum) unabsorbed	1/16	++++ ++++	++++ ++++	++++ ++++	++++ ++++	± −	− −
	1/32	++++ ++++	++++ ++++	++++ ++++	++++ ++++		
	1/64	++++ ++++	++++ ++++	++++ +++	++++ ++++		
	1/128	++++ +++	++++ +++	+++ ++	++++ +++		
Serum W (lymphogranuloma venereum) absorbed with steamed psittacosis virus	1/16	++++ ++++	+++ +++	+++ ++	++++ +++	− −	− −
	1/32	++++ ++++	++ +	− −	− −		
	1/64	+++ +++	± −	− −	− −		
	1/128	+++ +++	− −	− −	− −		
Normal	1/4	− −	− −	− −	− −	− −	− −
Saline		− −	− −	− −	− −	− −	− −

TABLE V

ABSORPTION OF A LYMPHOGRANULOMA VENEREUM SERUM WITH STEAMED LYMPHOGRANULOMA VENEREUM VIRUS

Serum		Lymphogranuloma Venereum Virus		Psittacosis Virus		Control Antigen	Saline
Treatment	Dilution	Unheated	Heated (100° C.)	Unheated	Heated (100° C.)		
Serum P. (lymphogranuloma venereum) unabsorbed	1/16	+ + + + + + + +	+ + + + + + + +	+ + + + + + + +	+ + + + + + + +	— —	— —
	1/32	+ + + + + + + +	+ + + + + + + +	+ + + + + + +	+ + + + + + + +		
	1/64	+ + + + + + + +	+ + + + + + + +	+ + + + +	+ + + + + + + +		
	1/128	+ + + + + + +	+ + + + + + +	— —	+ + + + + + +		
Serum P. (lymphogranuloma venereum) absorbed with steamed lymphogranuloma venereum virus	1/16	+ + + + + + + +	+ ±	+ + +	+ ±	± —	— —
	1/32	+ + + + + + + +	— —	— —	— —		
	1/64	+ + +	— —	— —	— —		
	1/128	— —	— —	— —	— —		
Normal	1/4	— —	— —	— —	— —	— —	— —
Saline		— —	— —	— —	— —	— —	— —

It has been shown that if a psittacosis antiserum made in the guinea-pig is absorbed with steamed psittacosis virus the corresponding antibody is removed, leaving the antibody to the labile antigen more or less unchanged, whereas absorption with the unheated virus removed both (Bedson, 1936). It was thought, therefore, that absorption of human lymphogranuloma venereum sera with steamed virus ought to remove their ability to react not only with the steamed homologous virus but also with both forms of heterologous virus, leaving them to react in an entirely specific manner. It was argued that the reaction with the unheated heterologous virus was apparent only and due to the heat-stable group antigen even in the unheated virus being available for reaction with the group antibody. Theoretically, either steamed psittacosis or lymphogranuloma venereum virus could be used for the absorption, and this, in fact, proved to be so.

Absorption Test

Psittacosis or lymphogranuloma venereum virus, prepared as already described (partially purified and steamed), was deposited from suspension by centrifugation for one hour on an angle centrifuge at 3,000 r.p.m., the supernatant discarded, and the deposit resuspended in the serum to be absorbed, suitably diluted. A concentration of eight times the titre was found satisfactory. Absorption was allowed to continue overnight in the refrigerator, when the virus was removed by thorough centrifugation (two hours on an angle centrifuge at 5,000 r.p.m.). The absorbed serum was then titrated against all four antigens, heterologous and homologous heated and unheated, in parallel with the unabsorbed serum.

Absorption with the control antigen was shown to have no effect on the antibody content of the serum. The results obtained in the absorption of two lymphogranuloma venereum sera, one with steamed heterologous virus and the other with the homologous preparation, are recorded in Tables IV and V.

In both cases the absorption has removed, or very considerably reduced, the ability of the serum to react with the heterologous virus heated or unheated, as well as with the heated homologous antigen, and left the major part of the antibody for the unheated homologous virus ; the test has been made specific. That this in fact is so is shown by the experiment recorded in Table VI, where the serum from a case of psittacosis has been absorbed with steamed virus ; again the test becomes specific.

Admittedly the procedure is laborious, and it is not suggested that it should be adopted as a routine in the serological diagnosis of lymphogranuloma venereum. It is, however, available in those cases where doubt exists.

The Frei Test

In the Frei test, as in the complement fixation test as usually done, the effective antigen is the heat-stable group antigen ; at any rate that is true of the routine Frei test made by us, in which the steamed lymphogranuloma venereum virus as used in the complement fixation test, but without any added antiseptic, has been employed. The antigen was used in double the dilution effective in the complement fixation test ; the dose employed was 0.1 ml. Readings were made at two and four or five days. The size of nodule produced was measured, and anything less than 0.5 cm. at the second reading was regarded as negative ; the extent of erythema was ignored.

The great majority of patients examined serologically were also submitted to the Frei test, and in recording the results of this test in Table VII

TABLE VI

ABSORPTION OF A PSITTACOSIS SERUM WITH STEAMED PSITTACOSIS VIRUS

Serum		Psittacosis Virus		Lymphogranuloma Venereum Virus		Control Antigen	Saline
Treatment	Dilution	Unheated	Heated (100° C.)	Unheated	Heated (100° C.)		
Serum M. (psittacosis) unabsorbed	1/16	++++ ++++	++++ ++++	++++ ++++	++++ ++++	− −	− −
	1/32	++++ ++++	++++ ++++	++++ ++++	++++ ++++		
	1/64	++++ +++	++++ ++++	++++ +++	++++ ++++		
	1/128	++++ ++++	++++ ++++	+++ ++	++++ +++		
	1/256	++++ +++	++++ +++	++ +	++ +		
Serum M. (psittacosis) absorbed with steamed psittacosis virus	1/16	++++ ++++	++++ +++	++++ +++	+++ +++	+ +	+ ±
	1/32	++++ ++++	+++ ++	++ +	+ +		
	1/64	++++ ++++	+ ±	± ∓	− −		
	1/128	++++ +++	± ∓	− −	− −		
	1/256	+++ +++	− −	− −	− −		
Normal	1/4	− −	− −	− −	− −	− −	− −
Saline		− −	− −	− −	− −	− −	− −

TABLE VII

CORRELATION BETWEEN FREI TEST, LYMPHOGRANULOMA VENEREUM COMPLEMENT FIXATION TEST, AND CLINICAL DIAGNOSIS

Clinical Diagnosis of Lymphogranuloma Venereum	Number of Patients	Lymphogranuloma Venereum Complement Fixation Titre						
		<1/4	1/4	1/8	1/16	1/32	1/64	1/128
Confident	28	0/0*	0/0	0/0	4/4	4/4	11/11	9/9
Possible	25	6/7	0/1	3/4	6/7	2/2	2/2	2/2
Doubtful	59	3/49	1/3	0/2	0/4	1/1	0/0	0/0

* Numerator gives the number of positive reactors and the denominator the number of patients tested.

an attempt has been made to show the relationship of the Frei test with the clinical and serological findings.

Two things strike one about these results. The first is the close parallelism between the Frei test and the complement fixation test in the clinically acceptable group, and the second is the number of patients suspected possibly of having lymphogranuloma venereum who have a strongly positive Frei reaction but little or no antibody in their blood. It is well known that the Frei test may remain positive long after clinical cure, whereas one would expect the antibody titre to fall when the infection became quiescent ; whether infection is ever eradicated or how often this occurs it is impossible to say. It seems, therefore, that those patients with a positive Frei test and little or no antibody are old cases of lymphogranuloma venereum in which the disease is now quiescent. And since the effective antigen in the Frei test material is the group antigen it follows that a positive reaction by itself does no more than indicate that infection with a virus of the psittacosis lymphogranuloma venereum group has occurred, but whether recently or not only the additional evidence supplied by the complement fixation test can tell us. The two tests should be used concurrently, and it seems justifiable to conclude that in a patient with symptoms at all suggestive of lymphogranuloma venereum a positive Frei test with a complement fixation titre of 1 in 16 or over indicates an active infection with this virus. In the clinically doubtful group there is one result which is out of keeping, that of a patient in whom the Frei test was positive and whose serum gave a titre of 1 in 32. This patient, a man, was first seen when he had a urethritis of one month's duration. The discharge was muco-purulent and, both microscopically and culturally, was free of pathogenic micro-organisms. He was married. No

clinical or laboratory evidence of lymphogranuloma venereum infection was found in his wife, and he denied extramarital venereal exposure. The Frei test made when the patient was first seen was doubtful, but when repeated three weeks later had become strongly positive. The complement fixation test on the first occasion gave a titre of 1 in 8 which rose to 1 in 32 three weeks later and, when tested after a further interval of a month, had fallen to 1 in 8. Treatment consisted of irrigation only, and the response was so satisfactory that when the third specimen of serum was collected he was clinically well. The results of the Frei test and the complement fixation test both suggest active lymphogranuloma venereum infection, and it is said that a primary lymphogranuloma venereum lesion in the anterior part of the urethra may produce symptoms of a non-specific urethritis. What is surprising is the transient nature of the infection, and this raises the question as to whether or not this may not have been infection with the related virus of inclusion blenorrhoea. An absorption test on the second specimen of serum might have settled this point ; unfortunately, there was insufficient of this pertinent specimen on which to do the test.

Intradermal Reactions with Psittacosis Virus

Reference has been made to the fact that the Frei test may be positive in psittacosis infections (Rake, Eaton, and Shaffer, 1941), but with the exception of some observations of Pollard and Witka (1947) intradermal tests with psittacosis virus in patients with lymphogranuloma venereum do not seem to have been made. Using steamed virus for this purpose the same result as with steamed lymphogranuloma venereum virus might be expected, since the effective antigen in both would be the group antigen. Parallel intradermal tests with steamed lymphogranuloma venereum

TABLE VIII

INTRADERMAL TESTS WITH PSITTACOSIS VIRUS IN PATIENTS SUSPECTED OF HAVING LYMPHOGRANULOMA VENEREUM

Clinical Appraisal of Condition and by Complement Fixation Test	Number of Cases	Intradermal Tests with Steamed Psittacosis and Lymphogranuloma Venereum Virus			
		Both Positive	Both Negative	Lymphogranuloma Venereum Positive Psittacosis Negative	Lymphogranuloma Venereum Negative Psittacosis Positive
Lymphogranuloma venereum...	16	13	0	3	0
Probably not lymphogranuloma venereum	23	1	20	0	2

and psittacosis viruses have been made in 39 patients suspected on clinical grounds of having lymphogranuloma venereum. The conditions of the test were as in the Frei test described above, and only induration of 0.5 cm. or more was considered positive. The results, which are given in Table VIII, show considerable concordance. The three patients with a positive reaction to lymphogranuloma venereum virus and a negative one to psittacosis all showed some reaction to the psittacosis antigen, but since the induration was less than 0.5 cm. it was recorded as negative ; the reactions were 0.3 cm., 0.3 cm., and 0.4 cm. None gave any reaction with the control (normal yolk sac) antigen ; in fact, only one egg-sensitive patient has been encountered in this work.

Attempts to Produce a Specific Skin Test Antigen

Experiments in which psittacosis virus from mouse spleen was tested by complement fixation after treatment with various reagents (Barwell, 1948) had shown that high dilutions of potassium periodate readily inactivated the heat-stable group antigen: fresh unheated virus suspensions, treated in the same way, still reacted with sera containing specific antibody. It was thought that this might provide a means of removing the group reactivity of skin test antigens. Preliminary tests with heated lymphogranuloma venereum virus showed, however, that reactions equal to those given by control antigens were obtained after treatment with 0.04 Molar KIO₄—that is, at least ten times the concentration required to remove complement fixing ability. This difference might be due to the fact that only a small proportion of group antigen is destroyed in the virus particle by periodate.

The earlier serological studies had also shown that dilute acids, like heat, yielded elementary body suspensions which reacted as the group antigen. This suggested that the specific component might be carried into solution in the presence of acid ; it was not possible, however, to demonstrate by complement fixation any activity in such extracts after they had been freed from virus particles. The possibility that solutions made in the same way might react specifically in the skin of infected patients was then investigated.

Technique.—Partially purified yolk sac suspensions of psittacosis and lymphogranuloma venereum viruses were made in saline from recently harvested material ; 0.1 N.HCl was then added to give a final concentration of 0.02 Normal. The pH of the mixture was between 3.0 and 4.0 and an immediate clearing was usually obvious. The tubes were placed at 37° C. for 15 minutes and then spun on an angle centrifuge at 4,000 r.p.m. for one and a half hours. The slightly opalescent supernatant fluid was removed and its pH adjusted to 7.4–7.6 with NaOH. A white or pale yellow precipitate began to form at a pH of about 5, and became floccular and usually copious as neutralization proceeded. It was removed by centrifugation. A single skin test failed to reveal any activity in this material. The clear supernatant fluids (acid extracts) were used undiluted as skin test antigens. The method was that described above for the Frei test.

Only a small number of patients has so far been available for this trial ; the results obtained with five cases of lymphogranuloma venereum and with one of psittacosis are shown in Table IX, in which are recorded the reactions to extracts of the two viruses as well as to the heated, diluted suspensions. It will be seen that the acid-soluble antigen of psittacosis virus fails to produce a reaction in the cases of lymphogranuloma venereum ; in each of them, however, the homologous extract elicits a definite response. In the one case of psittacosis the results of using these preparations were equally specific. Patients with lymphogranuloma venereum are again shown to react to heated suspensions of psittacosis virus, but it was not possible to demonstrate activity of a heated suspension of lymphogranuloma venereum virus in the patient who had recovered from psittacosis.

TABLE IX

ANALYSIS OF RESULTS OF SKIN TESTS AND REACTIONS TO LYMPHOGRANULOMA VENEREUM AND PSITTACOSIS VIRUSES

Patient	Clinical Findings	Comple-ment Fixation Test Titre	Skin Tests				
			Heated Suspensions			Acid Extracts	
			Lympho-granuloma Venereum	Psittacosis	Normal Yolk Sac	Lympho-granuloma Venereum	Psittacosis
T33986	Inguinal adenitis 4 weeks	1/64	6*	Not done	0	25	<5
T34684	Penile sore 6 weeks	1/16	5	5	0	17	0
H7265	Rectal stricture	1/128	++ Slough 10 mm.	12	0	20	0
H5779	Proctitis	1/32	8	15	0	25	0
T8930	Penile ulcer 1 month's duration	1/16	10	8	0	23	0
F.A.R.	Psittacosis 6 months pre-viously	1/64	0	20	0	0	15

* Figures indicate the diameter in millimetres of the area of induration observed 2 days after intradermal inoculation.

Summary

Titres of 1 in 32 or over in the complement fixation test made with steamed lymphogranuloma venereum virus and the sera from patients in whom the clinical findings are compatible with a diagnosis · of lymphogranuloma venereum suggest active infection with lymphogranuloma venereum virus.

As others have observed, the sera from human infections with psittacosis and lymphogranuloma venereum viruses show a very high degree of cross reaction in. the complement fixation test with antigens made from these two viruses. These cross reactions occur to titre, or almost so, with antigens in which the heat-stable antigenic component is the effective one, but to a lesser degree, particularly in the case of psittacosis sera, with the fresh unheated viruses; this difference is insufficiently great or constant to be diagnostic. Sera from human infections with psittacosis and lymphogranuloma venereum viruses contain specific antibody which can be revealed by removing the group antibody by absorption with steamed virus whether homologous or heterologous.

The Frei test, as usually done, also merely indicates that infection with a virus of the lymphogranuloma venereum psittacosis group has occurred.

A positive Frei test, together with a positive complement fixation test at a serum dilution of 1 in 16 or over, in a patient suspected of having lymphogranuloma venereum is good evidence of active lymphogranuloma venereum infection. Acid extracts of lymphogranuloma venereum and psittacosis viruses appear to give specific reactions when injected intradermally in human infections with these two viruses.

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THE PLATE VIRULENCE TEST FOR DIPHTHERIA

BY

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A method has recently been described (Elek, 1948) for the detection *in vitro* of toxin-producing organisms including *C. diphtheriae*. The present paper has two purposes. The first is the examination of a larger series of strains of *C. diphtheriae* with a view to establishing the reliability of the procedure, for if the method is to be of any practical value for clinical purposes it must demonstrate all toxin-producing strains within a reasonably short time. The second purpose is the standardization of the ingredients used so as to yield the best results and to ensure that the conditions of the test may be accurately reproducible in different laboratories.

Petrie and Steabben (1943) first suggested the use of nutrient media as a matrix for toxin-antitoxin reactions. They evolved a method for the detection of toxicogenic clostridia and briefly reported some experiments with diphtheria bacilli. Ouchterlony (1948) showed that when diphtheria antitoxin was incorporated in a serum ditch plate and a toxin-producing strain streaked across, several lines were produced, one of which was caused by the toxin. For the practical test of his *in vitro* method he incorporated various concentrations of antitoxin in serum agar plates, as did Petrie and Steabben (1943), and each strain was tested on a series of plates with falling antitoxin concentrations. The appearance of a halo around the inoculum was regarded as positive. Two series of strains were examined by this method and by the subcutaneous guinea-pig test. In the first series of 237 strains there was a discrepancy amounting to 8.5%. Of the guinea-pig positive strains 5.1% failed to give a halo, and 3.4% of avirulent strains gave false positives. In the second series of 308 strains the discrepancy was 6.2%, and included 4.2% false positives. Elek (1948) criticized Petrie

and Steabben's method on the grounds that a ring in a serum plate does not necessarily signify a toxin-antitoxin reaction and described a new technique using a medium better suited for toxin production. The antitoxin gradient was set up by means of a filter paper strip dipped into highly concentrated refined antitoxin. This system was found to be free from the zone effect and the theoretical considerations are dealt with in a later paper (Elek, in press). In a small series complete agreement was found between the *in vivo* and *in vitro* methods, and furthermore the results could be read within 24 to 48 hours compared with the 48 to 96 hours required by the method using serial plates containing graded quantities of antitoxin. Carter and Wilson (1949), using Elek's method but substituting human serum for horse serum, found absolute agreement with 200 strains between the *in vivo* and *in vitro* tests. Ouchterlony (1949) in a later paper reports the use of serum ditch plates. Readings were taken at 24-hour intervals for up to four days.

Concerning the constituents of a medium designed to give a good yield of toxin much information is already available. Almost all the work however was carried out with the classical Park-Williams No. 8 strain, and it is by no means certain that a medium giving optimal toxin production with it will necessarily meet the requirements of all other toxin-producing strains. Until about 25 years ago it was widely believed that only meat infusion media could be used for the culture of diphtheria bacilli. Davis and Ferry (1919) stated that it was impossible to obtain growth unless 0.2% meat infusion was present, and 10% was needed for toxin production. Wadsworth and Wheeler (1928) succeeded in producing toxin on peptone without meat infusion, but with various chemicals added. The addition of glucose to

infusion media as a source of energy was suggested by various authors (Park and Williams. 1896 : Smith, 1899 ; Locke and Main, 1928 ; Ramon, 1929 ; Hazen and Heller, 1931) and sodium lactate was introduced by Wadsworth and Wheeler in 1928. Pope (1932a) in a very careful reinvestigation of the effect of various carbohydrates and organic acids found that a concentration of 0.6% lactic acid was optimal. He used proteose-peptone for his media and the Park-Williams No. 8 strain for toxin production. He concluded that acclimatization to the medium was unimportant, but severe heat treatment, such as autoclaving of the medium, would destroy toxin production without interfering with growth. Pope and Healey (1933a and b) examined maltose concentrations and found 0.4% to be the optimum, 0.8% yielding decidedly less toxin. They found further that the best growth of the organism did not necessarily yield the maximum amount of toxin, but that this varied with the medium. They also established the very important fact that the initial presence of the toxin was without effect on growth and further toxin production. Finally they proved that toxin production was maximal, other things being equal, when growth occurred at the air/liquid surface. The results of these observations were incorporated in the medium previously described (Elek, 1948).

Experimental Work and Materials

Subsequent work has shown that successive batches of the medium showed some variation, and slight modifications have been made. Charcoal-clearing has been omitted, as filtering through paper pulp resulted in a sufficiently clear medium. The method of adjusting the reaction had to be standardized as this stage determines the precipitation of phosphates, and consequently the iron content of the medium. The preparation of the medium was accordingly modified as follows.

Twenty grammes of proteose-peptone ("difco"), 3 g. of maltose, and 0.7 ml. of lactic acid (B.P.) were dissolved in 500 ml. of distilled water. To this 1.5 ml. of 40% NaOH was added and the medium well shaken and heated to boiling point. The deposit was filtered off through filter paper and the reaction adjusted to pH 7.8 by adding normal HCl. Then 500 ml. of 3% agar (powder) were prepared in 1% NaCl and its reaction adjusted to pH 7.8. This was filtered through paper pulp in a Buchner funnel and then added to the fluid base. After distribution in 10 ml. quantities it was sterilized by autoclaving for 10 minutes at 10 lb.

To ensure an even thickness of the medium special compressed flint-glass petri dishes, 3½ in. in diameter. were used. As these are mould-compressed the bottoms are absolutely flat. and this combined with a standard amount of medium for pouring (12 ml.) gives an even thickness in all the plates. The plates were poured on a levelling board supported on three points and set horizontal with a spirit level. For routine purposes neither the mould-compressed plates nor the levelling board is absolutely necessary. Commercial refined diphtheria antitoxin globulins were used diluted with sterile carbol-saline (0.5% phenol in normal saline) to a concentration of 1,000 units per ml. This preparation of antitoxin contains 0.35% of cresol, but as this does not diffuse out it does not interfere with the reaction. In fact dilution with carbol-saline is useful as it reduces the risk of contamination. A filter strip measuring 60 mm. by 15 mm. sterilized by dry heat was moistened with the antitoxin by immersing it completely or dropping the antitoxin on to it. Plates were poured with 10 ml. of the medium and 2 ml. of normal horse serum and the filter strip was embedded in the still fluid medium leaving an unbroken surface for inoculation. Two brands of maltose were tried. One was an old batch without a manufacturer's name and the other was "difco." They gave identical re-

G = *gravis*
M = *mitis*
I = *intermedius*
█ = Results with batch A
▯ = Results with batch B
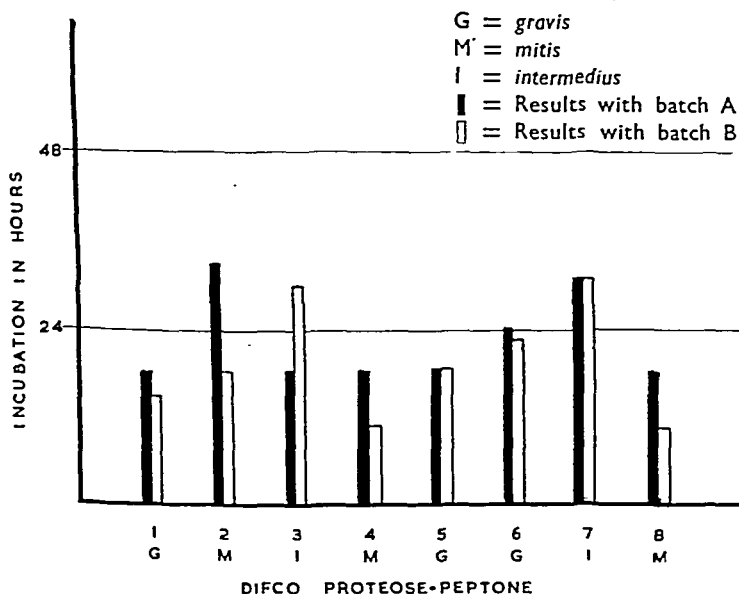
Fig. 1.—Comparison, on the basis of the time required for positive results, of two batches of medium (strains 1 to 8).

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within 48 hours. The general impression one gains is that neither is quite as potent as the "difco" proteose-peptone (compare Fig. 1). Evans peptone, however, is almost as good, and "difco" peptone comes third. "Eupeptone No. 1," "oxoid" and Witte's peptone are clearly unsuitable for the purposes of this test. Witte's peptone gave positives with only two strains within 48 hours (both *gravis*). The other two peptones gave no positives in two days, the time within which all virulent strains gave positive results with proteose-peptone. Evans peptone is probably suitable for the test, but testing with a much larger series is required. So far this has been done only with "difco" proteose-peptone.

Iron and Copper Content.—Locke and Main (1930) found that media usually used for toxin production contained about 1–4 mg. of copper per litre, and about 0.1 to 0.4 mg. of iron per litre. Pope (1932b) re-investigating the problem found that the addition of copper to media had little effect as long as it did not exceed 8 to 10 mg. per litre, which was the maximum tolerated. He also found that filtered medium was improved by iron, but media sterilized by heat did not require additional iron. Amounts of 0.5 mg. per litre were found to be definitely detrimental. The effect of iron on toxin production has been further investigated in recent years (Pappenheimer and Johnson, 1936; Mueller, 1941; Mueller and Miller, 1941). These workers found that the optimal amount of iron varied greatly with the composition of the medium and with the strain. No attempt was made in the preparation of the media used in the present experiments to control either the copper or the iron content, except by the use of glass vessels and the careful control of the precipitation of the phosphates. The iron contents of the broths prepared with various peptones were determined in case the reason for the divergent results lay in this. Table I shows the total iron content of the fluid base prepared with the six peptones tested.

TABLE I

IRON CONTENT OF BROTH MEDIA PREPARED WITH DIFFERENT PEPTONES

Peptone				Fe (mg./100 ml.)
"Difco" proteose-peptone		...		0.021
Evans ...	...	...	...	0.035
"Difco" ...	...	...	...	0.086
Witte's ...	...	...	...	0.050
"Oxoid" ...	...	...	...	0.046
"Eupeptone No. 1"		...	...	0.042

Except for the addition of agar these media were identical with the ones referred to above. Clearly the iron content does not explain the variation in toxin production although the best results were obtained with the two peptones showing the lowest iron values. Considerations of the iron content of the dry reagents (Table II) shows that little

TABLE II

IRON CONTENT OF DRY REAGENTS

Reagent	Fe (mg./100 ml.)
Agar powder	5.64
"Difco" proteose-peptone ...	3.93
Evans peptone	4.35

significance can be attached to small variations occurring in the final medium. It can be calculated that 1.5% agar corresponds to 0.084 mg. Fe. per 100 ml. For purposes of comparison this was left out of account partly because it represents a constant addition to all the media and partly as not all of it is in an ionizable form. The calculated amount of iron in 2% proteose-peptone is 0.078 mg. per 100 ml. as against the 0.021 mg. actually found. Thus, in the preparation of the medium approximately three-quarters of the iron is lost with the precipitation of the phosphates. A similar loss can be calculated in the case of Evans peptone. The essential fact is that media prepared in glass vessels will yield satisfactory results without an adjustment or even determination of the iron content so long as the precipitation of the phosphates is carried out in a standard manner.

Serum Enrichment.—The remaining variable is the added sterile normal horse serum. Early in the course of these experiments it was noted that the addition of horse serum improved the reaction. This effect may well be due to enrichment of the medium leading to increased toxin production, or the adjuvant effect may be upon the flocculation, or possibly both these causes may be operative. It was assumed that even though the antitoxin in the strip might contain all the necessary non-specific constituents for flocculation it would be unlikely that they would follow the same concentration gradient as the antibody globulins. On the other hand the addition of normal serum to the medium will yield an even concentration of any non-specific substances that may be required to intensify the flocculation lines. The views as regards the need for non-specific substances in flocculation reactions are somewhat contradictory, and the available information concerning their role and nature is insufficient for application to this test in a systematic way. It was decided, therefore, to examine the problem empirically.

Although, as has been shown, an exact reproduction of the base medium is relatively easily obtained, the addition of different batches of horse sera may materially affect results. This is clearly a considerable disadvantage for a routine test, since in the absence of knowledge of the various factors which determine the usefulness of the horse serum little can be done to obtain uniformity. Attempts to substitute bovine albumin for the horse serum were not successful. The sera used in the course of these experiments were commercially obtained No. 2 normal horse serum, heat treated, and issued for the preparation of
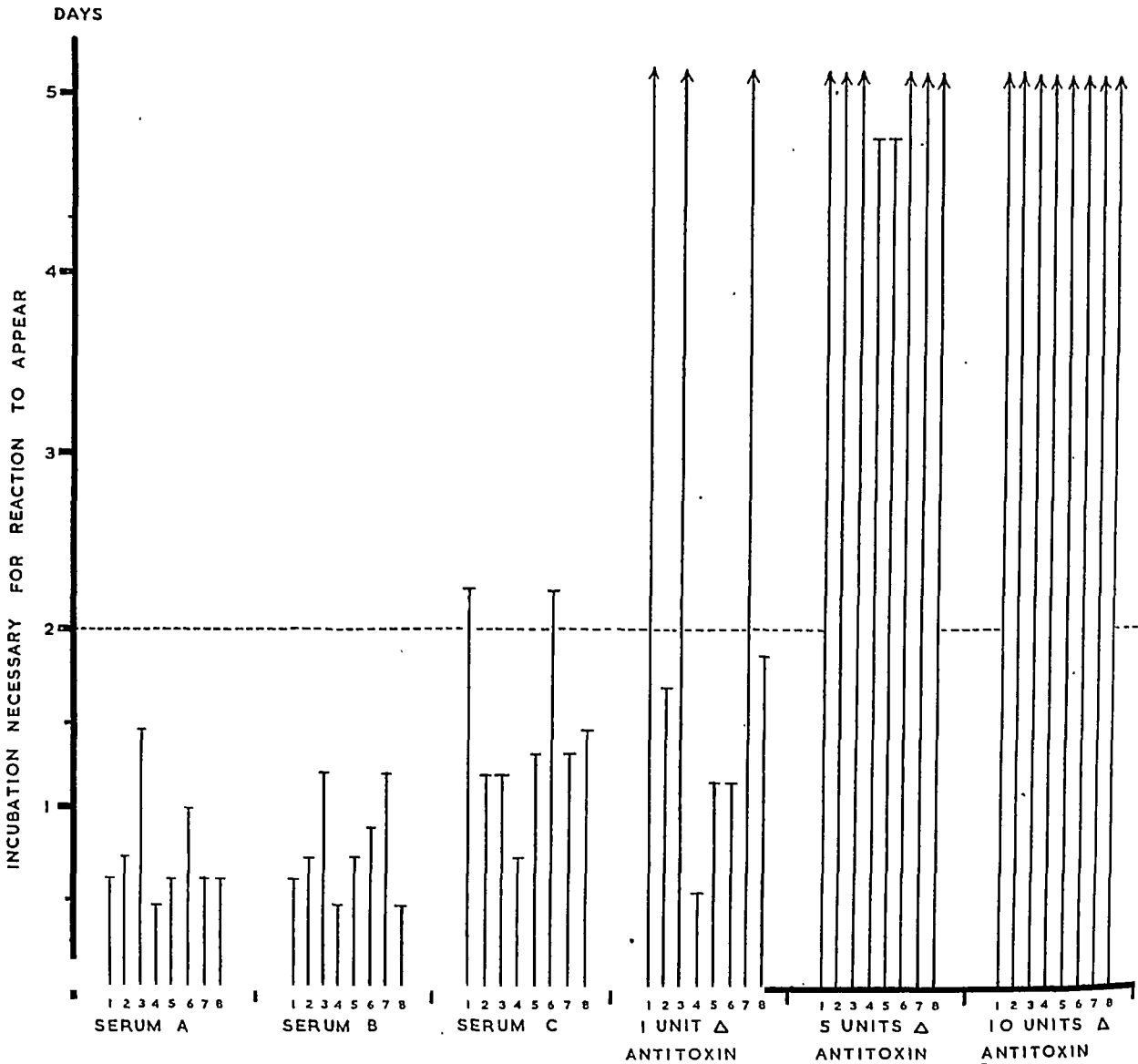
FIG. 3.—Effect of different batches of horse serum (A, B, and C) and the effect of adding diphtheria antitoxin to serum A. Base medium prepared with " difco " proteose-peptone. Strains 1 to 8 same as in Figs. 1 and 2.

media by the Wellcome Physiological Research Laboratories. The results obtained with three different batches of horse sera are shown in Fig. 3. Two of these (A and B) were No. 2 Wellcome brand sera and the third (C) was a normal horse serum obtained from a different source. The same eight strains of diphtheria bacilli were used as in the previous experiment. The base medium was the same batch for all, made with proteose-peptone. Readings were taken as before. Sera A and B did not differ in any material way, and both yielded positives for all the strains well within 48 hours. Serum C, on the other hand, required longer incubation for the lines to develop, and two strains failed to give positive results

within 48 hours, the lines becoming visible a few hours later. Clearly with this serum one would miss two virulent strains unless readings were taken after two days. It is hardly possible to conjecture why this serum was inferior to the others, but one possible explanation might be that it contained natural diphtheria antitoxin which interfered with the reaction. Normal horse sera of course vary in this respect, and in some unselected samples the titre may be quite appreciable. It seemed interesting to test the hypothesis whether a " bad " serum could be unsatisfactory on account of a high antitoxin level. To serum A varying amounts of crude horse antitoxin were added to yield concentrations of 1 unit, 5 units, and 10 units

per plate. As the volume per plate was 12 ml. this corresponds to 0.08 unit, 0.42, and 0.83 units respectively per ml. of medium. Fig. 3 shows the effect of the added antitoxin: with 1 unit per plate five strains gave positive results within 48 hours, but three strains, one *gravis* and two *intermedius*, failed to produce lines even in five days. Five units per plate almost completely inhibited the reaction, but two strains just managed to produce lines within five days. It is clear from this that a high natural antitoxin level will give an unsatisfactory serum for this test. On the other hand there is no evidence that this is the only reason for a serum proving unsatisfactory. It was found that the addition to serum C of purified diphtheria toxoid in amounts sufficient to over-neutralize any natural antitoxin did not improve it. This variation in the serum is at present difficult to overcome and represents the most serious disadvantage to routine use of this medium. Admittedly only small quantities of serum are required per plate, and each plate can be used for about six strains. Furthermore, a batch of serum once tested and found satisfactory can be kept in the refrigerator for long periods without apparent deterioration. The difficulty was the initial testing of the batch. At first a known strong toxin-producing strain was included on every plate, but this is obviously not satisfactory since weak toxin-producers can be overlooked in this way. Dr. A. T. Glenny suggested the use of a strain which he used for many years as a control in intracutaneous virulence tests. This strain, which he kindly let me have, was maintained in the freeze-dried state and has a fixed low virulence: in the ordinary skin tests it gives a reaction which can just be accepted as positive. On testing with the plate method it was regularly late in producing a line, and with serum A it required about 40 hours' incubation for a positive reaction to appear. Two further strains were encountered which showed similar behaviour, one a *gravis* and the other an *intermedius* strain. Both of these were reported as giving a very weak intracutaneous virulence test, and this had its counterpart in a weak line appearing only towards the end of the second day's incubation. At present this biological approach constitutes the only way of standardizing the serum. Once a batch is found satifactory it can be kept at low temperature, but it is advisable to include one of the weakly toxicogenic strains with each series of tests as a control.

The Reading of the Test.—The reading of the test presents no difficulties and requires no previous experience. The lines at their earliest appearance require oblique illumination and a hand lens. Good oblique illumination can be obtained by mounting two pieces of photographic paper between half-plate glass leaving a strip of about 1 cm. in the middle. This slit is held behind and parallel to the filter strip. By moving it slightly up or down in front of an electric bulb, a very early reading of the lines can be taken. However, a few hours later they become readily visible even by transmitted light. By the end of 24 hours the large majority of the virulent strains showed lines easily visible by transmitted light.

Comparison of *In Vivo* and *In Vitro* Tests

The final assessment of the method from the practical point of view lies in its usefulness when applied to a larger number of strains of *C. diphtheriae* and in its agreement with the accepted animal tests of virulence. When we examine the tests used as a routine for establishing whether a strain is "virulent" or not, we find that the test, subcutaneous or intradermal, is based on specific neutralization of toxin produced *in vivo*. The difference between the established virulence tests for diphtheria bacilli and the *in vitro* test described here, is only the difference between the conditions leading to toxin production. In other words, the question to be answered is whether it is possible to produce a nutrient medium catering for the individual requirements for toxin production of all the strains encountered and whether the test is sensitive enough to detect the presence of the toxin thus produced. The results of parallel tests carried out on 135 strains are shown in Table III, the same strains giving identical results by each method. These strains were mostly isolated and tested during the latter part of 1947 in London. The determinations of type and guinea-pig virulence were carried out independently of the plate tests by the bacteriologists who supplied the strains. Of the 67 strains described as *gravis* only one was avirulent, and this was obtained from the National Collection of Type Cultures. Of the 45 *mitis* strains, on the other hand, no less than 13 were avirulent, and all but one of these were isolated in the course of routine work. Of the 23 *intermedius* and untyped strains three were avirulent.

TABLE III
COMPARISON OF GUINEA-PIG AND PLATE VIRULENCE TESTS

Type of C. diphtheriae	Guinea-pig Test		Plate Test			Total No.
	Virulent	Avirulent	Positive in 24 Hours	Positive in 48 Hours	Negative in 58 Hours	
Gravis	66	1	61	66	1	67
Mitis	32	13	27	32	13	45
Intermedius and untyped ...	20	3	14	20	3	23

Discussion

The fact that the agreement in this series was absolute suggests that the sensitivity of the two tests is of a comparable order, and this is further borne out by the findings with the weakly toxicogenic strains already referred to. This is surprising indeed as the amount of diphtheria toxin required to produce a positive skin test in a guinea-pig is extremely minute, whereas the minimum amount of toxin required to produce a visible line in the plate is considerable and amounts to about 6 units. The two tests are, however, not comparable on the basis of the quantity of toxin alone since the conditions under which the organisms grow are vastly different. In the plate technique the organisms grow unhindered and a surplus of nutrient material designed for toxin production is available so that the accumulation of toxin is continuous.

From these observations it would seem natural to connect the time of appearance of the lines with the amount of toxin produced. Clinically and epidemiologically a method giving quantitative information of an organism's toxin-producing capacity would be of value. In model experiments using one filter strip soaked in toxin and the other in antitoxin, the position and angle of the line were related to the quantities used. With a streak of inoculum, however, visible growth precedes for a variable time the formation of the toxin-antitoxin line. The position of the line formed is dependent therefore on the actual shape of the antitoxin gradient at the time when toxin begins to be produced in an appreciable concentration. It is not practicable, for routine purposes, to measure the distance of the line from the filter-strip. On the other hand it was found that a given strain will produce a line within more or less the same time on repeated examinations, and it seems that the time can be taken to reflect a biological characteristic of the strain. Thus, although it is strictly speaking not a measure of the amount of toxin formed by a given strain, it still yields information of interest concerning the toxin-producing capacity.

Twenty-nine of the 135 strains tested were obtained from the National Collection of Type Cultures. Of these 27 were found to be virulent: 20 yielded positive plate tests within 24 hours and seven (26%) only after 48 hours' incubation. Of the remaining 106 strains, the majority of which were freshly isolated, 91 were virulent. Eighty-three of these showed a positive plate reaction within 24 hours, but eight strains required 48 hours' incubation for the positive reaction to appear. This confirmed the impression that old laboratory strains require rather longer incubation. The percentage of strains requiring as long as 48 hours' incubation to produce a positive reaction was 26% of the old laboratory strains, but only 8.8% of the freshly isolated virulent strains. Admittedly the numbers in each group are small. It is also interesting to note that all the freshly isolated *gravis* strains were virulent, and of these 27 were positive in 24 hours, leaving only two strains that needed two days' incubation. In the group of *intermedius* and untyped strains six were obtained from the National Collection of Type Cultures, but a further three were stock cultures. Of the remaining 14 freshly isolated strains 12 were virulent and two were not. Of the 12 virulent strains 10 gave positive results in 24 hours and the other two in 48 hours. Thus of the 88 freshly isolated virulent strains 81 (over 92%) were recognizable as such by means of the plate technique by the end of the first day. It was also observed that not only old stock cultures, but also freshly isolated strains can give a sluggish reaction if the streak on the test medium is made from a culture more than 24 hours old. It is essential, therefore, to use a young culture. The original culture may be on inspissated serum, blood agar, or tellurite medium.

It can be concluded from these data that the routine virulence test as performed by the intradermal method, and the *in vitro* test here described, give complete agreement as regards the 135 strains tested. This statement does not, however, invalidate the argument that true " virulence " of diphtheria bacilli is not synonymous with toxicogenicity. If there is a difference and toxin production is only one of the criteria of virulence, then we have at present no method available to assess the sum total of the various factors entering into the concept of virulence. It is clear, however, that toxin producion is a *sine qua non* of virulence. It is not possible to say whether a reversion to toxin production occurs in the case of avirulent strains. An interesting observation bearing on this question was made in the case of an old type culture strain (N.C.T.C. 322/Centry). This was received as avirulent, having been tested in 1920 by the bacteriologist who isolated it. It was then found that 4 ml. of a culture filtrate (further details not available) were non-lethal to guinea-pigs. This organism was found to be toxicogenic *in vitro* and this was confirmed by an intradermal animal test. Unfortunately the data of the original virulence testing were not sufficiently detailed and it is not possible to say whether a reversion to virulence occurred in this case.
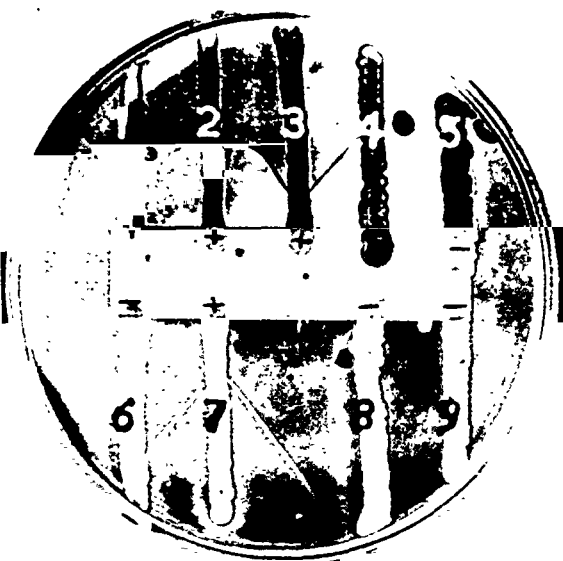
FIG. 4.—Looping of the toxin-antitoxin lines and the demonstration of non-specific turbidities. Strains 1, 2, 3, and 7 virulent *in vivo* (+). ・Strains 4, 5, 6, 8, and 9 avirulent (−).

FIG. 5.—Plate photographed after prolonged incubation and several days at room temperature showing secondary lines. Strains 1 and 3 are virulent, 2 is avirulent. Strain 1 shows two fine lines developing between the toxin line and the filter strip.

The rule of looping can be made use of when determining minimal toxin production by a strain. Fig. 4 shows a plate with nine strains of which Nos. 1, 2, 3, and 7 were virulent (÷) and Nos., 4, 5, 6, 8, and 9 avirulent (−) *in vivo*. Strain No. 2 is weakly toxicogenic and the lines produced by strains No. 1 and No. 3 show marked bending on approaching it. When the lines approach a strain that is devoid of toxin production they remain straight and cut the non-toxic streak ; that is, the line of strain No. 3 cuts across strain No. 4 which is non-virulent. It should be stressed, however, that the looping effect only applies when both lines are simultaneously produced. If one lags behind an angular joining of the lines occurs, as seen between strains No. 2 and No. 3, and this is due to the rearrangement of the antitoxin gradient around the line first formed. Any turbidity around the streak should be disregarded, since many organisms produce non-specific turbidities in serum agar media. Thus strain No. 7, which is virulent, produces a halo, but so does strain No. 8 as well although it is avirulent.

When the incubation is prolonged beyond 48 hours, or the plates left at room temperature for several days, very fine lines showing the typical arrowhead form may appear (Fig. 5, strain No. 1). These lines are definitely not due to toxin production as they appear with both virulent and avirulent strains. To avoid confusion the plate test must not be read later than 48 hours. The characteristics of these secondary lines are as follows: they appear late, usually after several days, they are fine, and may number two or three. Apparently they are due to diffusible antigens produced by diphtheria bacilli, the exact nature of which requires further elucidation.

In view of the secondary lines it is most important that the medium used in the plate test should be capable of demonstrating minimal amounts of toxin production in 48 hours, that is, before the appearance of secondary lines might lead to confusion. The medium, the preparation of which is here described, satisfies this requirement.

Summary and Conclusions

A method is described for assessing the value of various constituents in the preparation of media for the diphtheria plate virulence test.

Six different brands of peptone were found to vary in their suitability for the test.

The iron content of the media required no adjustment provided the preparation followed the routine described.

T

Batches of horse sera vary in their suitability for the test and the value of the serum used has to be established empirically.

A series of 135 strains gave identical results by the plate test and the intradermal guinea-pig test. The sensitivities of the two tests were found to be of the same order.

Over 92% of freshly isolated virulent strains gave a positive reaction within 24 hours with the plate test, and 100% were positive after 48 hours' incubation. Readings must not be taken later than 48 hours.

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THE LABORATORY DIAGNOSIS OF TOXOPLASMOSIS

BY

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A case of congenital toxoplasmosis has recently been seen in this hospital The patient, a girl aged 5 years, presented with hydrocephalus, bilateral pes cavus, and blindness. The eyes showed extensive retinochoroiditis thought to be characteristic of toxoplasmosis.   Radiographs of the skull showed areas of cerebral calcification. Two years before the birth of the patient the mother had suffered from an illness accompanied by a rash and headache, thought to be rubella.   Otherwise there was no family history suggestive of toxoplasmosis, and the mother was in good health. The clinical aspects of this case are being reported elsewhere.

Although cases of toxoplasmosis are being reported from all over the world, so far only three cases have been published in England.   In the case of Jacoby and Sagorin (1948) the serological confirmation of the diagnosis was obtained from Dr. Sven Gard, of Stockholm, and in the two cases of Farquhar and Turner (1949) the serology was done by Dr. Sabin, of Cincinnati. Owing to the apparent rarity of the disease few laboratories in this country deemed it worth while maintaining a strain of *Toxoplasma*, but as more cases are suspected on clinical grounds laboratory methods of investigation will have to be made available.   This communication is an account of the diagnostic laboratory tests at present in use and our experiences with them in the case mentioned above.

The laboratory methods available for the diagnosis of toxoplasmosis are the isolation of the organism and the demonstration of antibodies to *Toxoplasma* in the patient's serum. In addition, a skin reaction, similar to the tuberculin reaction, may be obtained in positive cases with an antigen prepared from the *Toxoplasma*. All these tests are associated with the names of Sabin and his associates, on whose writings we have freely drawn. The clinical pathology of the condition affords little diagnostic help, although in the acute phase an increase in cells and protein in the cerebrospinal fluid is sometimes seen (Sabin, 1942a), an increase in protein but not in cells being occasionally found in apparently healed cases. No specific blood changes or eosinophilia have been reported. In the present case both the cerebrospinal fluid and the blood picture were normal.

Attempts to Isolate Toxoplasma

A centrifuged deposit of the cerebrospinal fluid was examined microscopically for *Toxoplasma*, with negative results.   Part of the deposit was inoculated intraperitoneally and intracerebrally into mice. After one month, during which the mice remained well, they were sacrificed and emulsions of their brains and viscera were inoculated into further mice. This precautionary passage is necessary because sometimes primary inoculation of infected material causes no manifest disease, whereas material from such mice is capable of causing sickness when passaged to further mice.

In this experiment none of the mice became ill at any stage. In view of the long clinical history of our case and the normal cerebrospinal fluid this negative result was not surprising.

As *Toxoplasma* is apparently an obligate intracellular parasite, multiplying within fixed tissue cells, it is unlikely that an artificial culture medium will be elaborated for its isolation. In the present case, cultures were attempted from the cerebrospinal fluid on several occasions into Boeck and Drbohlav's, N.N.N., and Harding and Hawking's (1944) trypanosome medium, with negative results. Inoculation into developing eggs was also unsuccessful.

Serological Methods

So far, neutralizing (Sabin and Olitzky, 1937), complement-fixing (Warren and Sabin, 1942), and cytoplasm-modifying (Sabin and Feldman, 1948) antibodies to *Toxoplasma* have been described. The complement-fixing antibody appears to be different from the neutralizing antibody in that it is more heat stable and may appear later and

disappear earlier in the disease. Similarly, it is different from the cytoplasm-modifying antibody in that sera containing a high titre of the latter may be devoid of complement-fixing antibody. Cytoplasm-modifying and neutralizing antibodies usually seem to occur together, but whether they are in fact identical is not yet clear. For these reasons we deal with the three antibodies and their demonstration separately, despite the fact that what is called the cytoplasm-modifying antibody may be the neutralizing antibody reacting in a sphere other than the rabbit skin.

Sabin (1948) expresses dissatisfaction with the rabbit neutralization test on the ground that, while it is capable of demonstrating the presence of neutralizing antibodies in the serum under investigation, it is impossible to tell whether these were acquired as the result of the present clinical disease or as a result of previous inapparent infection. He also states that these antibodies may not be present shortly after infection has taken place or some years after the infection has burned out, so that a negative complement-fixation test may be misleading. The cytoplasm-modifying antibody test and the quantitative data yielded by it he regards as most valuable in deciding the state of activity in a given case.

The Rh strain of *Toxoplasma*, a strain virulent for mice, was obtained through the courtesy of Colonel H. E. Shortt, of the London School of Hygiene and Tropical Medicine. This is the strain originally isolated by Sabin in 1939 from a case of acute encephalitis (Sabin, 1948), and it has since been maintained by passage in mice. Blood from the patient and her mother was withdrawn by venepuncture and allowed to clot in the refrigerator. The serum was separated four hours later and divided into 1-ml. amounts. Half of these were immediately frozen solid and stored at $-60°$ C., while the remainder were inactivated at $60°$ C. for 20 minutes for use in complement-fixation reactions and thereafter stored at $-20°$ C.

Neutralizing Antibody. — Levaditi, Sanchis-Bayarri, Lépine, and Schoen (1929), working with *Toxoplasma* infection in rabbits, were unable to demonstrate any neutralizing antibody in convalescent serum, and concluded that the resulting immunity, which was absolute, was entirely cellular in origin. Sabin and Olitzky (1937), however, showed that there was a species specificity to toxoplasmosis. The majority of smaller animals, such as mice, guinea-pigs, and rabbits, died of the infection, and survivors did not show any demonstrable antibody. Rhesus monkeys, on the other hand, developed a non-fatal infection, and their

convalescent serum had the power to neutralize *Toxoplasma* when injected intracutaneously into the skin of a rabbit. Sabin (1941) showed that this neutralizing antibody also developed in human beings, and described a convenient method for its titration using the intracutaneous route in the rabbit. He also showed that the neutralizing antibody had no apparent *in vitro* effect on the *Toxoplasma*, as no agglutination or lysis could be detected in mixtures of immune serum and *Toxoplasma* suspensions. Sabin and Ruchman (1942), working with rhesus monkeys, showed that this neutralizing antibody had certain unusual characteristics. It appeared early in the disease and persisted for at least 15 months without any marked change in titre. They also showed that the antibody was of low titre and extremely labile. It was inactivated at $56°$ C. for 30 minutes and the titre dropped markedly if kept at room temperature or stored at $4°$ C. for a few days. They found that the only method of preserving the antibody was by freeze-drying or by keeping the serum at $-60°$ C. (This factor is of great importance when testing for neutralizing antibodies in the laboratory.) Sabin (1942a) showed that the rabbit skin neutralization test was a reliable method for the diagnosis of the disease in humans and that a significant number of patients in whom the disease was suspected clinically had demonstrable neutralizing antibodies in their serum. Serum from the mothers of these cases usually had neutralizing antibodies as well.

Heidelman (1945) obtained a weak to strongly positive result in neutralizing antibody tests in 63% of a series of cases presenting congenital retinochoroiditis, and in six out of seven mothers of infants with congenital retinopathy. Sabin (1942b), in a group of 151 selected individuals, children with nervous and ocular diseases and many mothers of such children, found neutralizing antibodies in the sera of 59. Also, of 15 children presumably infected *in utero* with *Toxoplasma*, he observed that 13 of the mothers gave a positive neutralizing antibody test. Similarly Johnson *et al.* (1946), reviewing presumptive cases of toxoplasmosis from the literature and their own centre, reported that of 45 patients with retinochoroiditis and serum neutralizing antibodies only seven failed to show radiological evidence of cerebral calcification. Further, taking 32 selected patients of various ages with central retinochoroiditis, active or inactive, they found that 20 possessed neutralizing antibodies in their sera. Crothers (1943) recorded nine children, belonging to five families, in which a clinical diagnosis of toxoplasmosis was

supported by serological evidence. All the children had cerebral calcification, and seven of them had inactive retinochoroiditis. The disease affected two children in two of the families, and the mother of one of the pairs had cerebral calcification. The presence of antibody in the sera of healthy siblings is common. In a study of a particular family with a toxoplasmic member Johnson *et al.* (1946) found neutralizing antibodies in seven out of a total of nine children.

In normal people and others with acquired retinochoroiditis Heidelman (1945) found evidence of neutralizing antibody in the sera of 10%. Choosing 58 normal people at random Binkhorst (1948) found antibody in the sera of six. Johnson *et al.* stated that four laboratory workers dealing with *Toxoplasma* research altered from negative to positive in their serum reactions, but no sign of illness occurred.

Rabbit Skin Method.—This test was carried ou. in the present cases by the method suggested by Sabin and Ruchman (1942). Serial tenfold dilutions of *Toxoplasma*-infected mouse brain in Tyrode's solution were mixed with equal quantities of undiluted test serum. These were allowed to stand at room temperature for 30 minutes. Two controls were used, one incorporating a negative serum, the other in which serum was replaced by Tyrode's solution. After standing, 0.2 ml. was injected intracutaneously into a rabbit's back which had been suitably prepared for inoculation. Erythema usually developed around the serum-*Toxoplasma* mixtures after 24 hours and persisted for two to three days. Erythema and induration going on to necrosis developed on the third to fourth day, being maximal in those areas with higher concentrations of *Toxoplasma*. Readings were taken on the fifth and eighth days. The rabbits usually died between the tenth and twelfth

TABLE I

EXTENT OF RABBIT SKIN LESIONS

Serum	Dilution of Infected Mouse Brain				Result
	1/20	1/100	1/1,000	1/10,000	
V.D. ..	+ + + +*	+ + +	+	--	Doubtful
Negative serum	+ + + +	+ + + +	+ + +	+	Negative
Tyrode control	+ + + +	+ + + +	+	--	Negative
Mrs. D.	+ + + +	+ +	--	-	Positive

* + + + + signifies erythematous indurated lesion with central necrosis on eighth day, an arbitrary end-point at 1/20 in the control series; + + +, + +, +, — represent varying degrees of reaction compared with this control.

days of generalized toxoplasmosis. The results of the test are shown in Table I.

Difficulty was experienced in repeating these results. Accordingly, several variations in technique were tried, and we found that *Toxoplasma*-infected mouse peritoneal fluid gave results which could be reproduced more accurately.

This test is undoubtedly of great value in diagnosis but presents many technical difficulties, and when only a few sera are being tested at a time and in the absence of a known positive control the interpretation of the results is made even more difficult. Professor C. P. Beattie, of the Department of Bacteriology, University of Sheffield, very kindly undertook to check these sera for us. He found that V.D.'s serum neutralized 100 rabbit skin doses, whereas Mrs. D.'s serum gave an indeterminate result.

Chick Embryo Method.—Using the technique of Warren and Russ (1948) we adapted the Rh strain to the chorio-allantoic membrane. This appeared to offer several advantages. First, it avoided the necessity for continual animal transmission ; secondly, it provided a source of supply of the complement-fixing and skin-test antigens : and lastly, it seemed to be a possible method of determining the neutralization index of a serum. analogous to that employed in the pock-counting method with herpes and vaccinia viruses. Some evidence was obtained that the present sera reduced the number of lesions on the chorio-allantoic membrane compared with several negative control sera, but again we encountered various technical difficulties. These facts are mentioned here as they seem to warrant further investigation.

Complement - fixing Antibody. — Warren and Sabin (1942) showed that specific complement-fixing antibodies developed in the rhesus monkey during convalescence, but that they did not persist longer than a few months. Obviously if similar antibodies developed in the human, the complement-fixing test would provide a method of diagnosis which would have the advantage of being rapid and would obviate the necessity for animals. The complement-fixing antibody appeared to be more stable than the neutralizing antibody in that it was not inactivated at 56° C. for 30 minutes and the titre persisted for a long time in the refrigerator. Warren and Sabin (1942) obtained fair correlation between positive human neutralizing sera and positive complement-fixation reactions, but the results were not entirely satisfactory. Of 43 sera with neutralizing antibody as many as 20 had no demonstrable complement-fixing antibody. This failure to find complement-fixing antibody may

have been due to several factors. In the first place the antigen, in this case prepared from rabbit brain, may have been too weak. Secondly, the physical conditions under which fixation was allowed to occur may not have been entirely satisfactory. Thirdly, the titre of the complement-fixing antibody might have fallen (none of the cases was acute) to a level at which it was no longer detectable. Warren and Russ (1948) have recently described a method of cultivation of *Toxoplasma* in the developing chick embryo and the production of a specific complement-fixing antigen from the chorio-allantoic membrane. Using this antigen they obtained results more comparable with those obtained with the neutralizing antibody test, and significant titres were found even in cases which had presumably been infected several years previously.

Complement-fixing Test.—Dr. J. Warren, of the Virus Research Department, Army Medical Department, U.S. Army, Washington, D.C., very kindly sent a sample of complement-fixing antigen, together with some hyperimmune guinea-pig serum as a positive control.

The method employed was that of Warren and Russ (1948). Sera were inactivated at 60° C. for 20 minutes. Serial twofold dilutions of test sera, from 1/2 to 1/256, were made in normal saline. Four units of antigen, previously titrated in the presence of two units of antibody, and two units of complement were added to each tube. Fixation was allowed to take place in the ice box (4° C.) overnight. The haemolytic system, made up of an equal volume of 3% sheep cells and two units of haemolysin in saline, was then added to each tube. Results were read after incubation at 37° C. for one hour; 100% fixation was taken as the end-point. The necessary controls, including a known positive serum, were set up with each test. Normal antigen was prepared from uninoculated chorio-allantoic membrane. The results obtained are shown in Table II.

TABLE II

Serum	Serum Dilution giving 100% Fixation		Result
	Toxoplasma Antigen	Normal Antigen	
V.D.	1/16	0	Positive
Mrs. D. ..	1/32	0	Positive
Positive control	1/128	0	Positive
Negative control	0	0	Negative

0 = No fixation at serum dilution of 1/2.

One of the many difficulties in any complement-fixation test is the interpretation of the results. In those cases where it is possible to test an acute and convalescent sample of serum and thereby demonstrate a rise in antibody as the result of infection, more significance can be attached to such an increase in antibody than to the antibody level of a single sample. In chronic or healed toxoplasmosis one is invariably testing single samples, taken in some cases many years after infection. In Warren's opinion (1948) a titre of 1/8 is doubtful and 1/16 positive. Furthermore, such titres have greater significance if there is clinical evidence to support the diagnosis. We have been able to examine five sera, three from cases with clinical evidence of toxoplasmosis and two of the mothers. In all five the complement-fixation titre was 1/16 or greater when first tested. The titre did not vary after repeated tests during the ensuing two months. We also examined 200 routine Wassermann sera in order to obtain some idea of the normal antibody level of the population of this country. Of the 200 tested, 175 produced no fixation at 1/2; 10 were anticomplementary, and 12 showed some degree of fixation (in three cases up to 1/12) with both normal and *Toxoplasma* antigens. Three sera, however, gave titres of 1/4, 1/8, and 1/16 respectively, all controls being negative. These cases are being further investigated.

On the other hand, a negative complement-fixation titre (1/2 to 1/8) does not necessarily rule out the possibilty of *Toxoplasma* infection. These low titres may be the result of past infection or may be entirely non-specific. Ruchman (1948) has shown that approximately 10% of normal individuals in the U.S.A. have demonstrable neutralizing antibodies in their serum. Frenkel (1948), in a survey of the population of the U.S.A., has shown that 20% of apparently normal people give a positive skin reaction with toxoplasmin. Figures for the incidence of complement-fixing antibodies are not yet available. Until a more detailed survey of normal people in this country has been made it will not be possible to assess the precise significance of a complement-fixation titre of less than 1/8, and for this reason it is advisable to test sera by all available methods before ruling out the diagnosis of toxoplasmosis.

Cytoplasm-modifying Antibody.—The demonstration of this antibody, as described by Sabin and Feldman (1948), depends on the ability of immune serum to modify the staining properties of living toxoplasmas. If a suspension of toxoplasmas from mouse peritoneal exudate is subjected to the action of methylene blue the parasites

take up the stain and at the same time their crescentic shape is altered towards the spheroidal. If, however, toxoplasmas are first incubated with immune serum and then methylene blue is added, a proportion of them, depending upon the strength of the antibody, retain crescentic shape and fail to take up the stain. In a positive case all the toxoplasmas are unstained in the higher concentrations of serum, except that a prozone may be seen in the greatest strengths, while all take up the stain when the antibody is sufficiently diluted out. By suitable dilution a titre of the serum may be ascertained at which 50% of the toxoplasmas are stained, which is regarded as the end-point of the reaction. Fig. 1 shows both stained and

Fig. 1.—Results seen in a cytoplasm-modifying test. The arrows indicate unstained toxoplasmas. × 300.

unstained toxoplasmas. The unstained ones are so feebly refractile that it is almost impossible to photograph them, and the photographs of Sabin and Feldman of unstained toxoplasmas do not, in our experience, represent the completely unstained crescents which we consistently see with immune sera.

Sabin and Feldman (1948) have shown that although the antibody is apparently very labile, in fact it is reasonably stable, and when potency has been lost it may be restored by the addition of normal serum. They describe an "accessory factor," different from complement, which is sparingly present in human serum and very labile, whose presence is necessary for the cytoplasm-modifying antibody to act.

For the performance of the test the toxoplasmas must be used within an hour of being removed from the mouse. The antibody works slowly at room temperature, but its action is complete after one hour at 37° C. Methylene blue must not be older than three to four days, and for immediate staining Sabin and Feldman recommend a highly alkaline preparation, either 3 ml. of saturated alcoholic solution of methylene blue with 10 ml. of alkaline soda-borax buffer solution of pH 11 (9.73 ml. of 0.53% Na_2CO_3 plus 0.27 ml. of 1.9% $Na_2B_4O_7.10 H_2O$) or a freshly prepared 0.25% methylene blue solution in the pH 11 buffer.

Four-day-old peritoneal exudates are used, because at this time they contain the greatest number of extracellular toxoplasmas and because they have not yet been affected by the immune response of the host. Immediately after removal from the mouse peritoneum the exudate is diluted 1 : 5 with heparinized saline or serum to prevent the deposition of fibrin. Serial dilutions of the serum to be tested are made using a normal human serum to provide "accessory factor," and to these are added equal volumes of *Toxoplasma* suspension. After incubation for one hour at 37° C. a drop from each dilution is mixed with half its amount of methylene blue and examined under a cover slip at a magnification of 475 for counting the numbers of stained and unstained parasites.

Adhering rigidly to these instructions of Sabin and Feldman, we were unable to make the test work at all, presumably for a variety of reasons, some of which were clarified. First, with the *Toxoplasma* suspension diluted 1 : 5 with saline there was apparently an insufficiency of "accessory factor," and this was corrected by making the dilution in normal human serum. Secondly, for some unexplained reason we found that methylene blue solutions used on the day of preparation, which was our original practice, stained the toxoplasmas so faintly that a reliable differential count of stained and unstained parasites was unobtainable. Next, it was found that the recommended alcoholic methylene blue solution produced a deposit in the *Toxoplasma* suspension which rendered the parasites uncountable. Lastly, as luck would have it, the first donor serum used for diluting purposes yielded preparations in which none of the toxoplasmas was stained, although repeated several times, and eventually we found that this donor had a cytoplasm-modifying antibody in his serum to a titre of 1/16.

Cytoplasm-modifying Test.—The technique finally adopted, which differs in detail but in no way fundamentally from that of Sabin and Feldman, is as follows.

Four-day mouse peritoneal exudate is taken immediately into an equal volume of normal human serum containing 100 units of heparin per ml. (with greater dilutions of the exudate we are unable to obtain sufficient extracellular toxoplasmas to permit of easy counting). To 1 volume of serial dilutions of the suspect serum in normal serum is added 1 volume of the exudate, and the mixtures are incubated in a water-bath at 37° C. for one hour. After removal from the bath, 1 volume of 0.25% methylene blue in *p*H 11 buffer is added to each tube and counts are made after standing for a further 10 minutes on the bench. The methylene blue must not be less than two days old, and we have found that it stains well up to 14 days.

Occasionally for no apparent reason the whole test is a failure, all the parasites staining, none of them staining, or all staining so faintly that no differentiation can be made. For this reason we always include with the titrations controls of *Toxoplasma* suspension to ensure that the methylene blue is working, and a known positive serum titrated out beyond its end-point.

When counting the preparations the unstained toxoplasmas are often very difficult to see, which probably leads to an error in favour of the stained ones. To avoid any prejudice the counter does not know which serum or dilution he is examining. Intracellular parasites stain whether immune bodies are present or not, and are excluded from the differential count.

The simplicity of this test, when all goes smoothly, is only offset by the necessity for maintaining a strain of living *Toxoplasma* in the laboratory. In acute disease and in recent cases Sabin and Feldman found titres up to 1/16,348,

FIG. 3.—Curves obtained by cytoplasm-modifying test (V.D.) made on three different occasions.

whereas when the history suggested an infection six to seven years previously titres of 1/16 to 1/64 were present.

Both Mrs. D. and V.D. showed the presence of cytoplasm-modifying antibody, the former to a titre of 1/128, the latter to a titre between 1/64 and 1/128. Figs. 2 and 3 show the curves obtained in each case when the test was repeated on three different occasions. They both show a prozone, the greatest number of unstained toxoplasmas not being seen until a dilution of 1/16. All the eight positive sera we have been able to test have shown this zoning phenomenon, and for this reason a full range of dilutions must always be set up, as a single tube test for presumptive diagnosis might fall in the prozone. It will be observed that although the total number of toxoplasmas remaining unstained varies from test to test the dilution at which 50% of them are unstained (the end-point of the titration) is remarkably constant.

Thirty control sera have not shown the presence of cytoplasm-modifying antibody, with the one exception of the donor whose serum was used as a diluent in our early efforts to make the test work and showed an antibody titre of 1/16 when diluted out' with other normal serum. Whether this is a non-specific reaction or denotes a previous infection is not clear, as the donor shows no clinical evidence of past infection and his serum is persistently anticomplementary.

FIG. 2.—Curves obtained by cytoplasm-modifying test (Mrs. D.) made on three different occasions.

Diagnostic Use of Toxoplasmin

Warren and Russ (1948) and Frenkel (1948) have recently described the use of toxoplasmin, a skin-test antigen, as an aid to the diagnosis of toxoplasmosis. Toxoplasmin can be prepared either from the chorio-allantoic membrane of the chick embryo or from *Toxoplasma*-infected mouse peritoneal fluid. In each case control antigens are prepared from normal uninfected tissues. After suitable preparation such antigens, which are in the nature of soluble antigens and are in fact the same as the complement-fixing antigens, have the property of producing a specific reaction when injected intracutaneously into sensitized people ; the reaction appears to be similar to the Mantoux reaction. Frenkel (1948) has shown that most patients with a positive neutralizing serum give a positive skin test with toxoplasmin, and that many cases in which the neutralization test has given a doubtful result also give a positive skin reaction. Toxoplasmin also appears to act as a " booster " to weak sera, as several were found to be positive at a later date. This did not occur with normal individuals inoculated with toxoplasmin.

Toxoplasma skin-test and control antigens were prepared according to the methods described by Frenkel (1948) and Warren and Russ (1948). The mouse peritoneal fluid antigen diluted 1 : 10,000 was used on V.D. and on three apparently normal children. An area of erythema 1 × 1 cm. developed on V.D.'s arm after 24 hours, but did not persist longer than 48 hours. The control children were completely negative. This was regarded as a doubtful result, and as such clearly needs further investigation.

The difficulties inherent in all the laboratory tests for toxoplasmosis are notorious. Earlier attempts to establish the rabbit neutralization test in this laboratory ended in failure mainly owing to the difficulty in interpreting the results. Similarly, the initial trials of the cytoplasm-modifying test were most disappointing, and only the clear-cut findings with the complement-fixation test led us to persevere until the snags had been eliminated.

From the nature of the case presented here attempts to isolate *Toxoplasma* were foredoomed to failure. But with the clear correlation between the neutralizing, complement-fixing, and cytoplasm-modifying antibody tests we feel we are justified on laboratory findings alone in making the diagnosis of a previous *Toxoplasma* infection. In the presence of a clinical history suggestive of the disease these findings assume added significance.

Summary

The laboratory methods available for the diagnosis of toxoplasmosis are the isolation of the *Toxoplasma*, the demonstration of neutralizing, complement-fixing, and cytoplasm-modifying antibodies, and the production of a skin reaction with toxoplasmin.

These investigations are described and discussed, and ways of avoiding some of the technical difficulties are indicated.

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UREA CLEARANCE TESTS

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CONTENTS

This paper has its origin in work done in a prisoner of war hospital camp in Siam. Under the prevailing condition of protein-starvation there the Van Slyke formulae for calculating urea clearance as a percentage of normal were found to be completely misleading. The only literature then available was a handbook of laboratory methods giving the bare routine of the test without indicating the source of factors in the formulae. Therefore an attempt was made to devise our own standards, including adjustment for the rate of urine excretion. When eventually I was able to obtain copies of the principal papers by Van Slyke and his colleagues (Austin *et al.*, 1921 ; McIntosh *et al.*, 1928 ; Möller *et al.*, 1928) it seemed of interest to compare our results with theirs.

The initial objective was to see if the method of adjustment for the rate of urine excretion used in Siam, or a modification of it, might provide a single generally applicable formula by which procedure could be both simplified and unified in place of the two in current use. This objective was achieved (see appendix), but in the course of the work I found myself forced to the conclusion that the Van Slyke urea clearance test is useless in its present form.

I. The Van Slyke Urea Clearance Test

The Van Slyke urea clearance test is the ratio of the amount of urea excreted per minute in urine to the concentration of urea in blood compared with the ratios observed in a sample of laboratory workers and since accepted in the textbooks as normal.

Depending on the rate of urine excretion it is computed by one or other of two formulae.

$C_s = U\sqrt{V}/B$ when V is less than about 2 (1).
$C_m = UV/B$ when V is greater than about 2 (2).
where V = volume of urine excreted in ml. per minute.
U = concentration of urea in urine in mg. N per ml.
B = concentration of urea in blood in mg. N per ml.

(Different units have been used at different times in the literature, but those quoted will be adhered to throughout this paper.)

C_s is called the " standard urea clearance at a urine excretion rate of 1 ml. per minute."

C_m is the " maximum clearance rate " observed at high rates of urine excretion.

The adjustment for the rate of urine excretion derives from the observation that the amount of urea excreted (UV) is approximately proportional to $\sqrt{V}$ when V is less than about 2 ml. per minute, and is constant when V is greater than that. The accepted normals are $C_s = 54$ with a range from 34 to 75 ; $C_m = 75$ with a range from 52 to 98. Observations outside these limits are supposed to indicate renal abnormalities.

II. Observations on Prisoners of War in Siam

The chemical aspects of the work in Siam and the conditions under which it was done have been described by Wilshaw (1947). Data are available for 65 men. Of these, 15, termed " normals," were observed as a check on what at first seemed the abnormal results being obtained for diagnostic purposes. The other 50, " patients," were men

sent by medical officers for observation. Available laboratory notes give clinical comments for only three cases. The case with the lowest blood urea level is recorded as one of renal colic for two years, and a case in the centre as renal colic for two years, the left kidney being palpable. The case with the highest blood urea level had blackwater fever ; this man was observed again, five weeks later giving the point at $y=0.1$, $u=-0.71$. The majority of patients were sent for a precautionary test of renal efficiency before operation for removal of a kidney containing stones, but were not expected to be renally deficient.

For each man we observed the volume of, and concentration of, urea nitrogen in the urine excreted during each of two successive hours, 10 to 11 and 11 to 12 a.m. (by sun time 8 to 10 a.m.) after a breakfast of rice and weak tea at 8 a.m. (6 a.m. sun time). Only one sample of blood was taken, about the middle of the two-hour period.

The precise amounts of protein in the diets are unknown to me, but were very low. Harvey (1946) quotes diets for a Singapore camp containing 25 to 86 g. protein per day ; amounts in Siam were roughly similar, but in this particular camp probably did not sink to the lowest limit recorded at Singapore. There was undoubtedly a good deal of variation. The group of normals included some cooks who could never refrain from helping themselves to extra ; some patients received supplementary diets ; some men only bought eggs with their available funds, and others spent it all on tobacco ; workers had more to spend than patients. (At that time an egg cost 25 sĕtangs. Men working for the Japanese received 25 sĕtangs a day, camp workers 10 sĕtangs, but non-working patients officially got nothing.)

Despite ersatz laboratory conditions the observations are of the same order of accuracy as those of previously published work. (The variance of deviations of log U from its regression on V and B was, for the normal group, 0.00263 compared with a corresponding figure for the data of McIntosh *et al.* of 0.00243, or 0.00174 if days on which urea was administered are omitted. Logarithms show variability proportional to the magnitude of the variate. Since the observations recorded here are on lower concentrations, observations in units of mg. per ml. would appear more accurate.

Urine urea concentrations show the same correlation with the volume of urine as was observed by Van Slyke *et al.* This can be described by a quadratic regression between the logarithms. Using this the urine urea concentration at any given rate of excretion can be estimated without arbitrarily dividing the data into two parts as with Van Slyke's method of adjustment. (For normal conditions, not stimulated by drinking variable quantities of water, as in the cases of the prisoners or the children, Table I, a linear equation may be sufficiently accurate.) Using the regression evaluated for the patients the log urine-urea-concentration for each man has been calculated as

$$\hat{y} = y + 0.49 \ (v - 0.174) + 0.25 \ (v^2 - 0.146)$$

where y, v, and v^2 are the averages of the two observations per man of log U, log V, and (log V)^2 respectively ; 0.174 and 0.146 are the overall averages of v and v^2 for the 50 patients. The effect is to adjust for the rate of urine excretion to the point where greatest average accuracy is obtained, namely at $v_0 = 0.223$ (V = 1.67 ml. per minute).

Since there was only one blood urea observation per man the internal correlation of V and B cannot be evaluated. Fig. 1 shows the bivariate distribution of $\hat{y}$ (=log U at V=1.67) and u (=log B). Obviously in these data there is no correlation of urine and blood urea levels between individuals.

The frequency distribution of observations of C_s is :

log C_s	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7
C_s	7.9	10.0	12.6	15.8	20.0	25.1	31.6	39.8	50.1
Frequency between above values	3	8	8	14	16	8	5	3	

If there were among the patients a group of renal defectives, and if the clearance ratio could detect these, we could reasonably expect a minor mode, or at least a longer tail, in the lower part of the range. These observations indicate a symmetrical distribution such as would be shown by a homogeneous group. On an arithmetic scale the tail would be at the upper instead of at the lower end.

Our records from Siam show some 45 additional observations on blood urea levels only, often with repetitions. Some of these gave very high values (particularly one just before death), but as they do not show anything unusual they need not be reported in detail. It is to be noted only that those exceeding 0.24 mg. urea-N per ml. did correlate closely with clinical observations. Although the evidence is incomplete there seems little doubt that patients represented in Fig. 1, with the possible exception of the one at B =0.34, were renally efficient. In the subsequent discussion they will be regarded as a sample of renally normal men ; the argument will not be impaired if a few were in fact diseased.

III. Comparison of Average Urea Concentrations

A comparison of urea concentrations and clearance rates observed in Siam with others recorded in the literature is given in Table I. Three main groups are represented.

(1) Residents of the U.S.A. who had, presumably, a fairly high protein diet; (2) Indians on a moderate to low protein diet; (3) prisoners of war on a very low protein diet.

The leading features as they affect the urea clearance test are immediately apparent. When enabled us to detect were that some prisoners had access to extra meat.

IV. Discussion of the Van Slyke Test

Much confusion appears to have originated in a failure to express explicitly the foundations of the test. Adjustments for variable factors have been derived on the basis of short term experimentally imposed variations within the individual, and it has been assumed that such adjustments were valid for comparison of permanent differ-

TABLE I

Source of Data	Number of Persons	Blood Urea—N mg. per ml.		Average Urine Urea —N mg. per ml.		Mean	C_s Range
		Average	Range	at $V = 2$	at $V = 1$		
U.S.A.: Austin et al. (1921), Möller et al. (1928): adults[1]	17	0.15	0.09–0.25	5.0	7.7	57	41–69
U.S.A.: McIntosh et al. (1928): children[2]	8	0.13	0.09–0.18	3.7	6.5	58	37–71
India, Bombay: Gokhale (1941)[3]	73	0.12	0.09–0.15	—	3.9[6]	32	17–44
,, ,, ,, ,, [4]	37	0.12	0.09–0.15	—	—	—	—
,, Mysore: Srikanhia et al. (1944)[5]	57	—	—	—	—	36	25–52
,, Tata Memorial Hospital: Pai (1945)	29	—	—	—	—	35	16–51
Prisoners of war, Siam: normals	15	0.17	0.12–0.26	2.5	3.7	23	15–34
,, ,, ,, ,, patients	50	0.16	0.07–0.28[7]	2.0	2.95	19	8–50

[1] Data for days when no urea was administered. Both limits of B shown by one person (FC); range of means per person 0.10 to 0.19.
[2] Adjusted to height = 175 cm., using regression of log U on log height = 1.5. Range of B for means per person 0.11 to 0.17.
[3] Group for which standard clearances were computed.
[4] Group for which maximum clearances were computed.
[5] Taken from data quoted by Pai.
[6] Calculated from average $C_s \times B$.
[7] Omitting two highest observations, 0.34 and 0.44.

diet is altered and the body is given time to readjust its balance to the new conditions, blood urea concentration is maintained at the same level as usual, in Europeans about 0.15 to 0.16 mg. urea-N per ml. The blood urea concentration does not respond to a permanent alteration of protein intake in the same way as it does temporarily to a dose of urea or to an exceptional meat meal. Therefore blood urea concentration is not in general an index of the amount of urea available for excretion, which, obviously, must balance with protein intake. This means that between individuals on permanently different diets urea concentration in blood tends to be approximately constant, whereas in the urine it is proportional to the protein consumed. Therefore in healthy persons the ratio depends on the diet, and a test case can give no useful information on other things unless it be related to a normal derived specifically from the average diet of the subject. In Siam the only abnormalities which the test

ences between individuals. In particular it has been assumed that the ratio of urine urea to blood urea makes allowance for variation in the amount of urea available for excretion, in other words, for variation in diet.

That assumption is essential for the validity of the test as it is usually applied, and yet I am uncertain whether or not it has been made consciously. More recent work seems to imply that the ratio is a characteristic worthy of study in its own right. This point of view will be discussed in section V. That it was eventually assumed to allow for differences in diet is made clear by the following quotation (Möller et al., 1928):

"In subjects with such renal loss the blood urea may, in fact, even be less than the normal average. Thus in experiments 10a and 20c blood urea nitrogen of only 10 to 12 mg. per 100 c.cm. is seen, despite the fact that both subjects showed only about 40% of

normal excreting power. Such results indicate the uncertainty attending interpretation of normal blood urea values in nephritic patients if the urea excretion rate is not also taken into consideration. It has in fact been common on our wards to find consistently normal blood ureas in nephritic patients who have lost 40–60%, of their renal function. Such patients are likely, either by choice or direction, to take diets low in protein ; and if they consume half as much as a given normal subject, other factors being equal, they will show about normal blood urea content."

This implies that these men have normal blood urea only because they are eating little protein, and that a normal man on their diet would have lower blood urea, so that the urine/blood urea ratio would be less affected than the urine urea concentration itself. Our observations show that a normal man eating little protein would show precisely the same urea clearance as these patients. The test cannot differentiate between the two.

A review in *Nature* (Srikanhia and Shamanna, 1945) of the Indian observations commented :

"The figures of Gokhale and of Srikanhia and Shamanna suggest that the Indian kidney has only about two-thirds the efficiency of its American counterpart."

The remark is typical of numerous others occurring throughout the literature. The urea clearance rate having become established as a measure of renal efficiency it has become customary to write, without thought that the measure may be faulty, "renal efficiency is low," when what is meant is that "the ratio of urine urea to blood urea concentration is low compared with the ratio observed in a small group of Americans." Our data show that observations of an extremely low urea clearance rate ($C_s = 10$, or 18% of the accepted normal) may have nothing whatever to do with renal inefficiency. The kidneys of all groups recorded in Table I were correctly performing their function of keeping excretion in balance with intake and so were fully efficient. Differences in their clearance rates measure only the protein diets of the subjects.

V. Methods of Deriving Efficient Diagnostic Criteria

The object of a clinical test is to discriminate between conditions of health and disease. There must be some criteria to which the results of a test can be referred in order to make a decision. In general there will be no hard and fast line between health and disease. In order to have a logical basis for deciding the levels at which

criteria should be set and for knowing just what subsequent tests may mean, it is necessary to consider the probability of a wrong diagnosis. The criteria should depend on some decision as to the relative seriousness of considering a person diseased when he is in fact healthy, or *vice versa.* The probability of the former can be assessed when the frequency distribution of the observation is known for a normal population similar in all respects to a population to be tested, except for occurrence of abnormalities of the kind being sought (Neyman's error of the first kind). The probability of the reverse will depend on how far the diseased condition is removed from normality, and consequently can only be assessed for specified degrees of abnormality (Neyman's error of the second kind).

If only one characteristic is observed the problem is merely to fix the point on a single scale where the probabilities of either error are reasonably balanced. But when two or more characters are observed we have a choice of numerous types of region which may be demarcated with equal probability of an error of the first kind. We have to consider which of these regions will maximize the power of the test to discern the abnormality in question.

To illustrate : let us assume that the 65 observations depicted in Fig. 1 form a representative sample of urea concentrations in urine and in blood for renally normal persons in the camp where they were observed. Suppose we decide to accept a 5% risk of an error of the first kind, that is of declaring a person abnormal when he is not. The problem is to demarcate a region of Fig. 1 containing 5% of the normal population in which an abnormal observation is likely to lie. To do this we also require evidence of the region (or regions) in which abnormal observations may occur. It has been noted that all the patients definitely diagnosed as renally defective had blood urea (N) greater than 0.24 mg. per ml. Therefore the only line we can reasonably draw on present evidence is the vertical line B. (The criterion for its position is that it is 1.6 times the standard deviation of u from the general mean, that is at a point estimated to mark off the upper 5% of the population on the hypothesis that it is normally distributed.) On that criterion the test is based on blood urea concentration alone without consideration of U (or $\bar{y}$). In the absence of more comprehensive evidence it is at least a reasonable criterion, since a high blood urea level is undoubtedly direct indication of disease. A criterion based on the urine urea concentration

alone would reject observations below the line A. The evidence of Table I indicates that this is primarily a criterion of diet, an indication which is consistent with the relative position of the normals (Fig. 1) who, being wage earners, would be in a better position than many of the patients to obtain supplementary food. Any line not parallel to A or B entails combination of both variates.

The procedure of the Van Slyke urea clearance test is to demarcate regions by lines at 45° to either axis.* For a 5% risk of error of the first kind this would be line S (which, it may be noted incidentally, would reject the blackwater fever case after he had returned to health).

The reasons for selecting this region can well be described as little more than a " hunch " that it might be informative. It is at first sight reasonable to suppose

FIG. 1.—Bivariate distribution of y and u for 65 prisoners of war. The full lines are drawn at distance 1.645 times the standard deviation from the general mean, $y = 0.375$, $u = -0.794$. Standard deviation of $y = 0.140$, of $u = 0.154$, and across diagonals about 0.14. The dotted line corresponds to $C_s = 34$, which is the lower limit for normal standard clearances as formerly given.

that when renal function begins to fail some increase in the blood urea level may be accompanied by a decrease of urea excretion relative to protein intake and blood urea concentration, and that the failure may be more quickly detected by considering both simultaneously than either one alone. There is, however, no *a priori* reason to suppose that the two effects occur in inverse proportion.

A method to determine the combination of two or more characters which may best discriminate between two groups has been described by R. A. Fisher (1936 ; 1946). Clearly in this case, since U has been shown to be closely linked to protein nutrition, quantities of protein consumed would need to be introduced as a fifth variate in addition to U, V, B, and body size. The efficient discriminant function depends on the relative errors of

observations on each variate as well as on their physiological relationships.†

The following considerations make it seem unlikely that a function of these five variates will be appreciably more effective to detect renal deficiency than will observation of blood urea levels alone. Either urea excreted must balance protein consumed, or blood urea must be increasing. If the kidneys are not functioning efficiently, presumably the excretion required to balance intake may be achieved by a higher concentration in the blood. If the patient in that way maintains a stationary position his urine must be normal for his diet (if it can be assumed that excretion in sweat is not materially increased), and therefore the condition is shown by a high blood urea level only. If the urea excreted is less than the intake, either the balance not excreted must be very small or blood urea must be increasing rapidly. It seems unlikely

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*As has been noted, the observations $\hat{y}$ in Fig. 1 are log U adjusted to $v_0 = 0.223$. The estimates of log U_t at $V = 1$ are given by

$$y_t = \hat{y} + 0.49 v_0 + 0.25 v_0^2 = \hat{y} + 0.1217$$

consequently contours of $C_s = U_t/B$ in Fig. 1 are given by equations

$$\hat{y} = \log C_s - 0.1217 + u$$

† Since the first draft of this paper was written (December, 1947) I have learnt that suggestions to apply these ideas to clinical medicine have also been made by others. A paper for presentation to a conference of the Royal Statistical Society in October, 1948, by R. B. Fisher, was entitled " An Application of Discriminants to Clinical Chemistry." So far as I know this is not yet published, nor do I know anything of its content. Analogous work in the U.S.A. has also been reported.

that determinations of urea in urine may be sufficiently accurate to detect the first of these conditions. In the second no refinement on observation of blood urea alone is likely to be required. Nevertheless the possibilities merit further investigation.

Addis proposed observation of the clearance ratio when supplementary urea was administered, and this is the test chiefly considered by Cope (1934). We had no means in Siam of artificially increasing blood urea concentrations and are not in this paper concerned with circumstances thus created except as they affect interpretation of the data of Möller *et al.* Presumably diet would not affect the response of normal subjects to extra urea ; and, if it can be shown that renally defective individuals react differently, this test might be of some use when referred to observations on the same individual "at rest" rather than to fixed standards.

Some observations by Van Slyke (1947) suggest that observation of the slope and curvature of the regression of log U on log V at low urination rates might be informative. This will, however, be very susceptible to differing abilities to evacuate more or less completely, and artificial methods might have to be used.

A more important defect in the procedure used to set criteria for the urea clearance test is illustrated by Fig. 1. The limits accepted as normal were simply the highest and lowest ratios observed in a casual sample of 18 subjects who seem to have been mostly workers in the laboratory. Our sample of 15 normals (about the same size of sample) was drawn in a similar way, being workers nearly all from one hut. Following the same procedure our lower criterion would have been given by a line through the point D and parallel to S. This would have resulted in classing 27% of our patients as renally deficient, which would have been manifestly erroneous. The reasonable explanation is that workers probably had better food than many patients. Analogous differences occur between the original laboratory sample of Van Slyke *et al.* and patients subsequently tested who, being suspected of renal defect, would often have been given protein-low diet. The lower criterion derived in that way, and since 1928 accepted as the dividing line for test purposes, is shown by the dotted line in Fig. 1. It would condemn 95% of those we observed, including all the normals, as renally defective.

The moral is that observations from which a clinical criterion is to be derived must be properly representative of the population to which the test is to be applied. Furthermore, in order accurately to assess the probability of error the sample must be large, say, 500 to 1,000 persons. Confidence limits derived from the *t* test of small sample theory are not satisfactory for setting control limits (Deming and Birge, 1938). The labour required will be small compared to the waste in applying tests whose reliability is unknown and undefined. It should be noted too that the essential requirement is observations on a large number of persons, not many observations on a few. Single observations per person would serve, but duplicates would be preferable to control gross accidental errors, and to obtain estimates of sampling variance within and between persons, say V_e and V_p. The error variance of a mean of n observations on one individual is then given by $V_n -V_e/n$; and the efficient number of observations to make in any given circumstances will depend on balancing the cost of n observations against value of consequent accuracy.[*]

VI. Summary

Observations on prisoners of war in Siam show that when diet contains little protein the blood urea concentration is still maintained at normal levels while urea excreted in urine is greatly reduced, as of course it must be to balance with the amount of protein consumed. Underlying the Van Slyke urea clearance test is the assumption that, by observing the ratio of urea in urine to its concentration in blood, allowance is made for the effect of diet on urine urea. That assumption is shown to be false. Consequently the urea clearance test as generally used without reference to diet is not a trustworthy measure of " renal efficiency " and may be completely misleading. Men with efficient kidneys were observed to have a " standard urea clearance " as low as 10, or 18% of the accepted normal.

The reasoning which led to setting misleading criteria for this test is discussed. It seems that observations on urine urea are unlikely to be of much use in testing for renal efficiency, but the possibilities should be further investigated. Methods for doing so and for deriving efficient criteria for clinical tests in general are indicated.

The observations on prisoners of war were made in the pathology laboratory of the camp at Nakom Patom, Siam, from January to July, 1945, by Major A. T. H. Marsden, R.A.M.C., pathologist, Malayan

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[*] Mr. B. G. Greenberg, research graduate in medical statistics, Institute of Statistics of the University of North Carolina, who kindly read the first draft of this paper, points out that sequential testing might profitably be used in clinical tests. This means that the probability of error is assessed after each observation. If the first observation indicates reasonable certainty it is accepted as final, but if it leaves more than the permissible margin of doubt then further observations are made until an answer is obtained at the required confidence level. (The theory is expounded in several papers by Barnard and others and in *Sequential Analysis of Statistical Data*.)

Medical Service, who was director of the laboratory, and by Major R. G. H. Wilshaw, chemist, Department of Agriculture, Malaya. The writer was at the time working in the laboratory as a biologist, but has been concerned with these data only as a statistician. 1 am indebted to the observers for putting their ingeniously obtained data at my disposal without restriction.

I should like further to record my appreciation of the courtesy shown by Professor G. R. Cameron, Dr. H. D. Barnes, and Dr. D. D. Van Slyke in discussing this work with an interloper led by circumstances of fate into a field not his own. Dr. Barnes brought to my attention the Indian work, which has therefore been added since the original draft of this paper, and provides welcome corroboration of work done under difficulties which might otherwise have been suspected of throwing doubt on their freedom from systematic error.

Dr. Van Slyke has brought to my attention several other papers on the effect of dietary protein and urine volume on urea clearance (Cope, 1933 ; Van Slyke *et al.*, 1934 ; Farr, 1936 ; Longley and Miller, 1942 ; Van Slyke, 1947). It may, however, be helpful to note that most of them appear to deal with comparatively short periods on altered diets and to consider only the ratio urine/blood ureas without attempting to follow alterations in urine urea and in blood urea separately. It seems that an abrupt change of diet rapidly alters blood urea concentration which returns to normal only gradually, hence the correlation between blood urea and urine urea noted by Van Slyke, but not shown among the prisoners. The full effect of altered diets on the ratio is therefore not apparent in short-term experiments. There is also a hint, in the observations of Van Slyke and in those on the prisoners of war, that stimulus from temporarily increased blood urea (either by administration of urea or by blackwater fever) may result in subsequent depression of blood urea. If so, it adds another reason for regarding the orginal data of Van Slyke as unsuitable for determination of criteria to be used in a test without that stimulus. These points require further investigation.

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APPENDIX

Relation Between Urea Concentration in Urine, Rate of Urine Excretion, Urea Concentration in Blood, and Body Size

From statistical analysis of the data of Van Slyke *et al.*, and from observations of the prisoners of war, the following conclusions were reached.

The relation of urea concentration in urine (U) to the rate of urine excretion (V) may be described by an equation of the form

$$\log U = A - \log (a + V)$$

or, more empirically, by

$$\log U = a - 0.48 \log V - 0.3 (\log V)^2$$

Curvature shown by different individuals varies considerably. The constants given are average values which may serve sufficiently well, when the number of observations on a person are too few to determine his own characteristic curve, to estimate urine urea concentrations for comparison with others. Adding regression on body height, a convenient equation to estimate urine urea concentration ($\hat{U}$) at any fixed urination rate (V_0) and height (H_0) is

$$\log \hat{U} = \log U + 0.48 (\log V - \log V_0) + 0.3 (\log^2 V - \log^2 V_0) - 1.5 (\log H - \log H_0)$$

where U, V, and H are observed values.

The two curves used by Van Slyke *et al.* to estimate urine urea concentrations at fixed urination rates are a reasonable approximation to the actual curve, but the consequent partitioning of data into two parts when such estimates are required is a nuisance and inefficient. Their "augmentation rate" is artificial. It does roughly indicate a point where the curve approaches close to its asymptote and beyond which it may be considered to be straight, but it is not a satisfactory statistic for comparison of different curves.

The hypothesis of Möller, McIntosh, and Van Slyke (1928a) that, at a given rate of urination, urine urea concentration is proportional to body size appears to be justified. Their further opinion that no accuracy is gained by trying to estimate surface area from weight as well as height is also confirmed. Their procedure for doing this is, however, circuitous and confusing. It would simplify computations and show better exactly what is being done if adjustment were based explicitly on height instead of on a supposititious body area.

When the blood urea level is temporarily increased by administration of urea or by a heavy meat meal, urine urea is increased nearly but not quite in proportion ; actually in proportion to about the 0.85th power of blood concentration. But for variations occurring with normal diet the relationship appears to be less close. Between different persons on variable diets there is no appreciable correlation.

THE SIGNIFICANCE OF THE UREA CLEARANCE

BY

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By the courtesy of Mr. H. Fairfield Smith we have had the privilege of examining in advance of publication his paper " Urea Clearance Tests." This paper appears to touch on three separate questions: (1) What does the clearance purport to measure? (2) What factors affect the clearance? (3) What is the diagnostic and prognostic significance of the clearance in renal disease? Inasmuch as the available data appear to have been only partially available to Mr. Smith, the writers venture to add the following comments on these questions.

What Does Urea Clearance Measure ?

The urea clearance measures the efficiency with which the kidneys remove urea from the blood stream, when due correction is made for the effects of low rates of urine flow. Studies on man and animals have shown that the urea clearance measures the rate of filtration of urea in the glomeruli minus the rate of urea reabsorption by the tubules (Van Slyke et al., 1934, 1935 ; Smith, 1937 ; Chasis and Smith, 1938 ; Shannon, 1936).

Factors Affecting Clearance

Like all physiological functions, the urea clearance varies under different physiological conditions, particularly those which affect the renal blood flow (Van Slyke et al., 1934, 1935) and the urine volume (Van Slyke, 1947 ; Möller et al., 1928 ; Dole, 1943). Consequently the clearance of a given subject may vary by as much as ±20% of his average under the usual conditions of daily life (Möller et al., 1928). The variation of the clearance with urine flow has been studied (Van Slyke, 1947 ; Möller et al., 1928 ; Dole, 1943) and shown to be due to variations in tubular reabsorption of urea that are predictable by the simple diffusion laws (Dole, 1943). High protein diets have been shown to increase renal blood flow and urea clearance in dogs (Jolliffe et al., 1931), and partial protein starvation has been shown (Cope, 1933) to decrease the clearance in men, a finding which is confirmed by Mr. Smith's observations.

Variability is shown by other physiological functions, measurement of which is nevertheless of value in assessing clinical conditions: e.g., the metabolic rate, body temperature, and pulse rate vary with physical activity, excitement, etc. Such variability does not invalidate the measurement of these functions, or of the urea clearance, in assisting to estimate the condition of a patient.

Renal disease affects as a rule chiefly the rate of filtration, which decreases as glomerular destruction advances, although in advanced nephritis increased permeability of the tubules appears to add its effect by increasing urea reabsorption, particularly when urine volume is low (Van Slyke, 1947). Damage to the tubules by nephrotoxic substances (Richards, 1929 ; Lucké, 1946), or by renal ischaemia, such as occurs in severe and prolonged shock (Phillips et al., 1946 ; Van Slyke, 1948 ; Phillips et al., 1948) appears to make the tubules more permeable to back diffusion of urea into the blood, and lowers the clearance by increasing urea reabsorption. In all these conditions the urea clearance has been found of value in assisting the appraisal of the extent of renal damage, and the course of progress either towards recovery or fatal renal failure.

The Diagnostic Significance of Urea Clearance in Renal Disease

Neither the urea clearance, nor any other physiological measurement, should be asked to serve as the sole criterion to discriminate between health and disease. The clinician using such a test must evaluate the results in terms of all known causes of variation, physiological and pathological. As the increase of metabolic rate with muscular or digestive activity or excitement does not invalidate its use in the thyroid clinic, so the decrease of the urea clearance with protein starvation or shock (Phillips et al., 1946 ; Van Slyke, 1948) does not invalidate its utility in following the course of renal damage or disease, when proper allowance is made for the effect of extra-renal influences.

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Studies of a considerable number of cases of renal disease of different types, some of which were observed over periods of years, have led (Van Slyke *et al.*, 1930) to the following conclusions:

"In acute hemorrhagic nephritis fall of the urea clearance to as low as 10% of normal was found not inconsistent with apparently complete recovery. . . . In all these cases which recovered or improved, however, the blood urea clearance began to rise within four months after the acute onset. . . . Of the different features of (advanced renal) disease that were followed, the blood urea clearance proved to be the most closely related to the onset of final renal failure. The renal function, measured by the clearance, could apparently remain indefinitely at 10% of normal without uremia ; but when it fell to 5% uremia occurred, and was usually fatal. Exceptions to the immediately fatal outcome were found in acute cases, which can recover if the functional depression does not last too long, and occasionally a terminal case, in which functional fall was partly due to factors, such as desiccation, other than destruction of renal tissue. In such a case treatment, particularly saline and glucose injections, may both improve the general condition and somewhat increase the urea clearance, although the added lease of life appears to be at most a few months."

These observations have been confirmed and extended in subsequent studies continued to the present time in this clinic, and will be presented in a future publication.

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THE INTERRELATIONS OF THE SERUM PROTEINS IN LIVER DAMAGE, WITH SPECIAL REFERENCE TO THE THYMOL TEST

BY

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Maclagan (1944a) demonstrated that phenol and certain derivatives when dissolved in barbiturate buffer at pH 7.8 produced a turbidity with human sera. He observed that under specified conditions sera from normal persons gave a negligible turbidity, and the most marked turbidities occurred with sera from patients suffering from parenchymatous liver damage. The concentrations of phenol or phenol derivative required to produce a given turbidity with any serum diminished as molecular weight increased, but at the same time solubility in an aqueous buffer diminished to such an extent as to limit the range of homologous phenol derivatives that could be used conveniently without the addition of extraneous substances to enhance solubility. As a result of his investigations Maclagan chose thymol as the most suitable derivative, and defined the conditions for carrying out the test and the degree of turbidity which might be regarded as outside normal limits. Maclagan (1944c) never claimed that his test was specific. It is none the less striking that sera in which the highest thymol readings are recorded are predominantly those from patients suffering from liver damage.

In 1947 Maclagan and Bunn employed electrophoresis to investigate the contributions of the individual components of the serum protein mosaic to the thymol turbidity test. They observed that turbidity occurred when γ-globulin from hepatitis serum was added to the thymol reagent and that human albumin from normal persons prevented this reaction. Albumin separated by electrophoresis from the serum of patients suffering from infective hepatitis did not prevent the reaction. They observed, further, that γ-globulin separated from the serum of normal persons would produce turbidity with the thymol reagent, but that the minimal concentration required was greater than γ-globulin from the serum of patients suffering from hepatitis. Neither normal α- and β-globulin, nor α- and β-globulin separated from the serum of patients suffering from hepatitis, gave turbidities with the thymol reagent, either before or after sensitization of the reagent with cephalin ; α- and β-globulin tended to sensitize the reaction of the thymol reagent with γ-globulin.

Kunkel and Hoagland (1947), studying the reaction of normal and pathological sera, demonstrated that when a serum giving a markedly positive reaction is extracted with ether the thymol reaction is markedly reduced, the example quoted in their paper showing a reduction from 22 to 9 units. They examined sera by electrophoresis before and after flocculation with the thymol reagent, and showed that such changes as there were affected the β-fraction principally. Finally, in six sera they examined, in which the thymol reaction was above 25 units, the lipid content was 100% higher than in six sera with a thymol reaction below 15 units. Their findings stress the significance of the lipid content of the serum and imply that the β-globulin is the active participant in the test.

Cohen and Thompson (1947) produced further data which they interpreted as indicating that β-globulin was the active agent in the thymol test.

The experiments of Wunderly and Wuhrmann (1947) in which γ-globulin was added to normal serum, thereby producing turbidity with the thymol reagent, seem to support Maclagan's conclusion that γ-globulin of itself can be responsible for the production of turbidity with the thymol reagent. Maclagan (1944b) pointed out that lipids might increase the sensitivity of the reaction and, indeed, he demonstrated that the precipitate from

the reaction contains cholesterol, phospholipid, and protein.

The fullest understanding of the alterations in the circulating proteins which result in positive thymol turbidity tests may throw light on the bio-chemical lesions underlying some forms of failure of liver function.

Material

In the past years I have been accumulating observations on the circulating proteins in the sera of patients suffering from liver failure. From this material the selected data seemed par-ticularly suitable for the study of the relationship of the thymol turbidity reaction to the nature of the proteins circulating in conditions primarily involving the liver.

The material may be divided into two groups: one, from patients suffering from, and recovering from, acute attacks of infective hepatitis; the other, from patients suffering from chronic progressive liver failure.

The β- and γ-globulins which formed the bases of special study were separated by electrophoresis from the sera of two patients suffering from acute hepatitis in whom the thymol turbidity values ranged at 32 and 36 units respectively.

Methods

The thymol turbidity tests were carried out according to the technique laid down by Maclagan (1944b).

The electrophoretic analyses were carried out in the apparatus designed by Tiselius (1937), us'ng the 11-cm. analytical cell, and in a barbiturate buffer of 0.1 ionic strength and pH 8.6 and in a phos-phate buffer at 0.2 ionic strength and pH 8.0. All protein concentrations were adjusted to 2.0% and dialysed against successive changes of buffer before analysis.

Ultracentrifugal analyses were carried out in the oil-driven centrifuge designed by Svedberg.

Nitrogen was estimated by the standard micro-Kjeldahl technique and cholesterol by a modifica-tion of the method of Schoenheimer and Sperry (1934).

Results

Acute Infective Hepatitis.—The proportion of γ-globulin in the group of patients with acute infective hepatitis is shown plotted against thymol units in Fig. 1.

The ratio of albumin to γ-globulin and of albumin to $\beta + \gamma$-globulin from the same group is shown plotted in Fig. 2. There appears to be poor
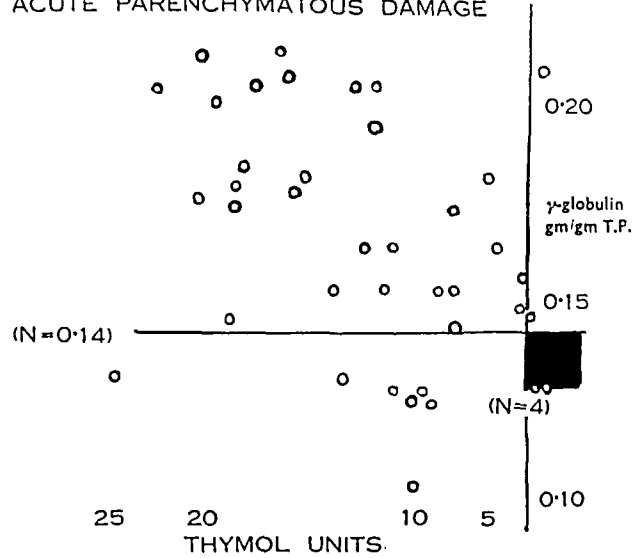
FIG. 1.—Chart showing γ-globulin (as percentage of total protein) plotted against thymol turbidity in acute infective hepatitis.

correlation between the γ-globulin concentration or the $\beta + \gamma$-globulin concentration and thymol units, but there is a definite correlation between the ratio of albumin to globulin and thymol units.

From two sera the $\beta + \gamma$-globulin fractions were separated and pooled. They were then divided
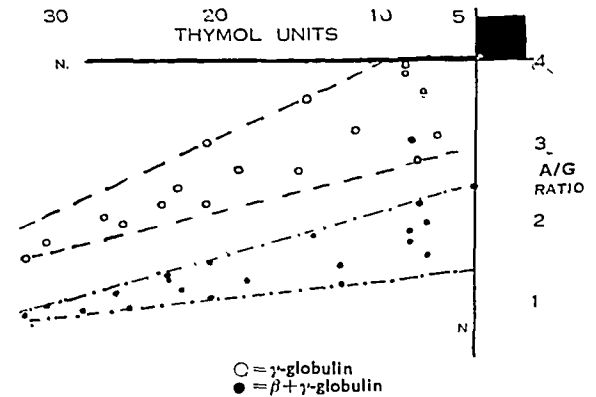
FIG. 2.—Albumin/γ-globulin and albumin/$\beta + \gamma$-globu-lin ratios plotted against thymol turbidity in acute infective hepatitis.

into aliquot portions and diluted with an equal volume of saline in which had been dissolved vary-ing concentrations of crystalline human albumin.

The results of the thymol turbidity test on the solutions so obtained are shown in Fig. 3. The thymol turbidity in units is plotted against the ratio of albumin nitrogen to $\beta + \gamma$-globulin nitrogen. It will be seen that the ability of the globulin to form a turbid solution with the thymol
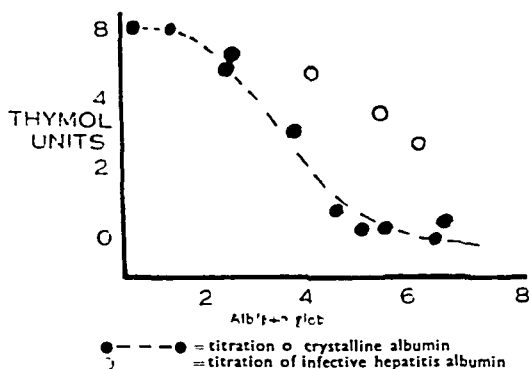
FIG. 3.—Effect of albumin in inhibiting positive thymol effect of β + γ-globulin in infective hepatitis with (a) normal crystalline albumin and (b) albumin from infective hepatitis.

reagent could be inhibited by pure albumin and that the extent of inhibition increased in proportion to the ratio of albumin to globulin. As has been previously recorded this reaction is not species specific (Martin, 1948), parallel effects being obtained with bovine and horse albumin. Three points are also shown of experiments in which albumin, separated from a case of infective hepatitis, was substituted for normal albumin.
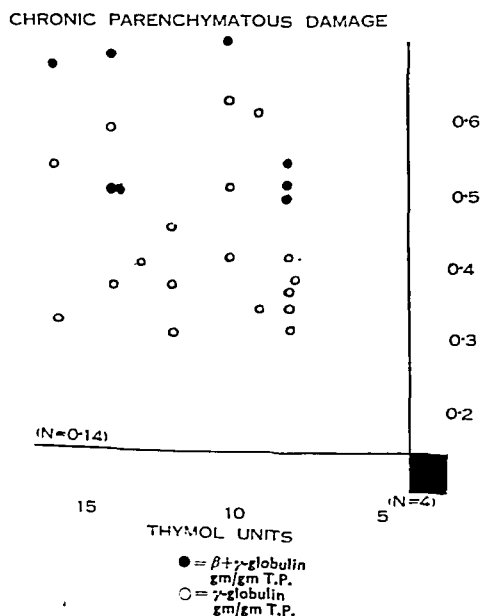
FIG. 4.—γ-globulin and β + γ-globulin (expressed as percentage of total protein) plotted against thymol turbidity in chronic liver disease.

Chronic Liver Damage.—γ- and β ÷ γ-globulin are plotted against thymol units in a group of patients suffering from chronic liver damage in Fig. 4, and the ratio of albumin to γ-globulin and to β ÷ γ-globulin is shown in Fig. 5.

FIG. 5.—Albumin/γ-globulin and albumin/β ÷ γ-globulin ratios plotted against thymol turbidity in chronic liver disease.

In contradistinction to the findings, shown in Figs. 1 and 2, neither approach seems to show any significant correlation between protein values and thymol units.

In the course of analysis of the cases of chronic liver damage, two were observed in which spontaneous precipitation of the γ-globulin took place during electrophoretic analysis.

The precipitation took place in the descending limb only after three hours when there was effective separation of the individual components of the protein mosaic and it is clearly confined to the γ-globulin. Spontaneous precipitation of this type has never been observed in the serum of patients with acute hepatitis.

Results in Non-hepatic Cases.—" False " positive reactions had been observed occasionally with the thymol reagent during the investigation of cases of hyperglobulinaemia, in which clinical and histological evidence did not suggest any liver damage and other biochemical tests did not support a diagnosis of hepatic insufficiency. These " false " positives were rarely above 10 Maclagan units. Ultracentrifugal analyses from some of these cases have been carried out and demonstrate that in certain instances there are molecules

present having abnormal sedimentation constants. Fig. 6 shows an analysis of such sera from a case of reticulosis (A), from a case of advanced liver damage (B), and, for comparison, an analysis of serum from a normal person is shown (N).

The presence of molecules with unusually high They can occur independently of any gross increase or deviation of lipoid in the serum.

The part which lipids play has been stressed by Kunkel and Hoagland (1947). Table I is an analysis of five sera in all of which there was raised cholesterol, inversion of the albumin
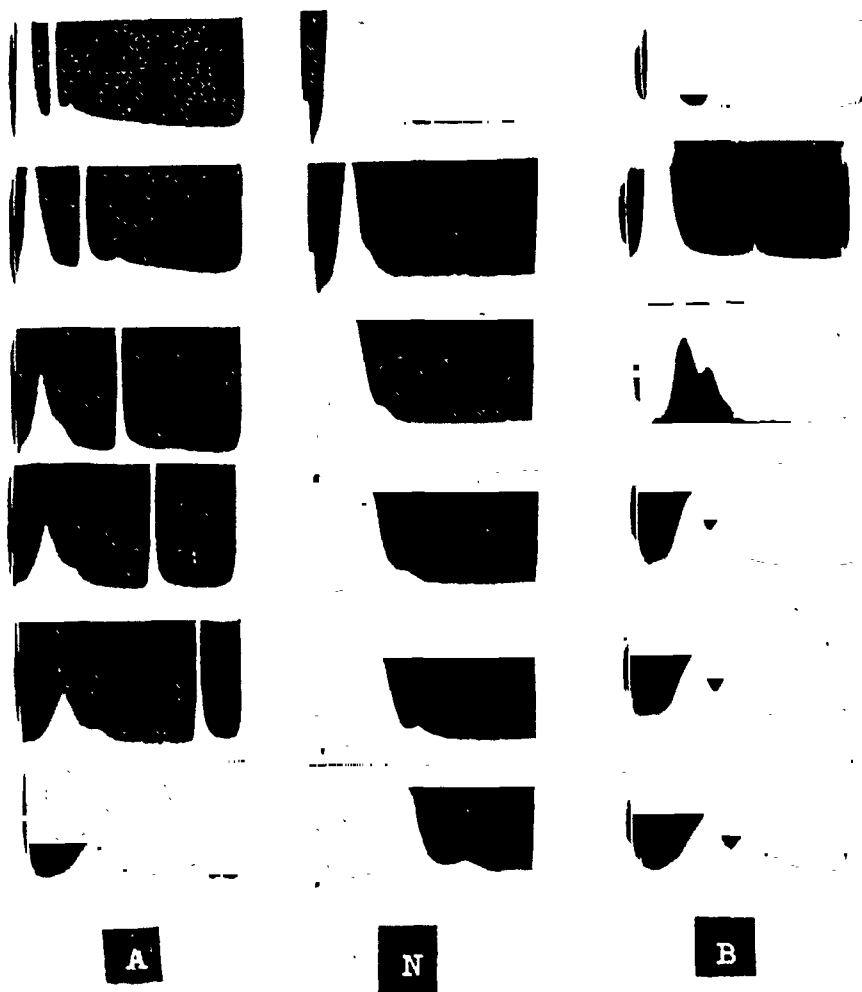
FIG. 6.—Ultracentrifugal diagrams showing fractions with high sedimentation constants in serum from a case of reticulosis (A) and advanced liver disease (B). Normal serum (N) is shown for comparison.

sedimentation constants can be seen in both serum A and serum B. They are not visible to any appreciable extent in normal sera.

These " macromolecules " were associated with the γ-globulin fraction in the sera studied. It must be stressed that they are not a constant component of the γ-fraction in liver disease, nor do they predominate in the γ-fraction when they do occur.

globulin ratio, and a normal reaction to the thymol reagent. Maclagan (1944b) had already shown that the presence of cholesterol increased the sensitivity of the test. It is clear from this table that considerable increases of circulating cholesterol may occur in the presence of inversion of the albumin globulin ratio without producing abnormal turbidities.

TABLE I

Case	Total Protein (g./100 ml. Serum)	A/G	Cholesterol (mg./100 ml. Serum)	Thymol Turbidity Units
1	3.5	0.3	405	3.0
2	4.2	0.33	450	2.0
3	4.4	0.25	437	2.0
4	5.2	0.34	470	3.0
5	6.2	0.5	375	2.0
Normal	7.0	1.25	200	up to 4.0

Discussion

The observations presented suggest that, in normal sera, the inability of the thymol reagent to produce more than trace turbidities is due to the interaction of normal albumin with γ-globulin, for, as Maclagan (1947) has demonstrated, neither α- nor β- globulins are capable themselves of flocculating with the thymol reagent. The marked turbidities produced in acute infective hepatitis seem to be correlated with the relation of circulating albumin to globulin rather than to the relative increase in the amount of circulating γ-globulin. Replacement experiments, demonstrating that the addition of normal human albumin to globulins separated from positive sera inhibits turbidity, lend added support to this contention. Similar experiments carried out with albumin separated by electrophoresis from sera of patients suffering from acute hepatitis suggest that in these, at any rate, there are, in addition, qualitative alterations in the circulating albumin which reduce its ability to inhibit the thymol reaction.

It is in acute hepatitis that the highest thymol turbidity readings are commonly encountered, though the disturbance of the albumin globulin ratio is rarely so marked as in chronic liver damage. The evidence suggests that in acute hepatitis qualitative defects in the circulating albumin are important.

The level of thymol turbidities in chronic liver damage bears little relation to the progress of the disease ; nor, in spite of the fact that gross inversions of the albumin globulin ratios are common, is there any correlation with the thymol turbidity readings.

The observation of spontaneous precipitation of the γ-globulin during the examination of the serum of two cases of advanced chronic liver damage suggested the presence of γ-globulins inherently unstable in themselves. The demonstration of molecules of abnormal sedimentation rate in the circulating sera of other cases of chronic hepatitis lends added support to the contention that in certain of these patients aberrant globulins contribute to the production of turbidity with the thymol reagent.

These macromolecules are demonstrable occasionally in sera from patients with diseases in which there is no evidence of primary liver damage, the sera frequently giving turbidities above the normal range. The presence of these molecules may be regarded as contributing to a " non-specific reaction." It must be stressed that these macromolecules are rarely present in high concentration and that their presence is by no means constant nor confined to sera from patients with liver damage.

Summary

Fresh evidence is put forward to support the view that the abnormal thymol turbidities associated with liver damage depend on a disturbance of the intimate relationships of the albumin and globulins in the serum.

Replacement experiments suggest that qualitative defects in albumin are important in the production of the reaction in acute hepatitis.

Abnormal globulins contribute to some positive reactions which are regarded as " non-specific."

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FAECAL FAT VALUES ON PRESENT BRITISH DIETS

BY

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During a series of faecal fat analyses on samples of dried faeces (Soxhlet method) it was noted that the " normal " total fat content was usually within the range of 10% to 15%, of which some 50% was split fatty acid. These values are significantly lower than those encountered in similar hospital patients before 1940, and can readily be explained by the reduced fat content of the present diet. They do, however, lead to a consideration of the effects of the present low fat intake on conditions usually associated with fatty stools.

It is appreciated that an analysis of pooled three-day samples of faeces is desirable, but such collections are unpleasant, difficult to handle, and, in the majority of cases, not practicable. Hence the usual hospital practice of utilizing a single morning specimen has been followed. It is important, however, that any comparison of normal and pathological findings be made on patients receiving similar diets, particularly with regard to the intake of fat.

Fairley (1936) showed that in 56 out of 70 cases of tropical sprue the total faecal fat exceeded 25% of the dried faeces, with an overall average of 39.1% of total fat, of which 64.8% was split.

It will be seen that in the present series of 10 cases of tropical sprue the average total faecal fat was 34.2% of which 70.6% was split fatty acid, and

in three cases of idiopathic steatorrhoea similar figures were obtained.

Normally, not more than 25% of the dried faeces should be fat, of which not more than 25% should be unsplit. When the total fat intake is low, split fat is almost completely absorbed, and even in health unsplit fat may form a higher proportion of the faecal fat, since some of it corresponds more or less to " starvation " faecal fat which may be regarded as fatty material excreted into the bowel or formed by degeneration of cell tissue, and not as a food residue (Harrison, 1947).

In the present somewhat limited series the average normal fat content of the dried faeces was 13.1%, with a range of from 7.5% to 17.7%. Splitting was somewhat low, the average split fat being only 54.3%.

Summary

Although the normal total faecal fat and fatty acids in the stool have been somewhat diminished by restrictions in diet, the rule that normally not more than 25% of the dried faeces should be fat is still a sound generalization.

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TABLE

FAT CONTENT OF DRIED STOOL

Condition	No. of Cases	Total Fat (%)			Split Fat (%)		
		Minimum	Maximum	Mean	Minimum	Maximum	Mean
Normal	20	7.5	17.7	13.1	29.1	86.4	54.3
Tropical sprue ...	10	24.2	45.7	34.2	61.8	82.0	70.6
Idiopathic steatorrhoea	3	31.1	46.3	38.2	63.8	75.4	69.6

VIBRATORY MOVEMENT IN THE CYTOPLASM OF ERYTHROCYTES

BY

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In 1890 Browicz described an intracellular vibratory movement in human erythrocytes, which he believed to be a pathological condition, from four cases of anaemia. It was found in one case of pernicious anaemia, and was stated to disappear following a remission ; it was also found in two cases of malignant disease. He ascribed it to abnormal reactions between pathological erythrocytes and their plasma. In his view it was not a vital phenomenon since it persisted at room temperature when leucocytic activity had subsided, and he stated that it continued for at least 24 hours.

Cabot (1901) made several references to this motion. He stated :

" If we focus carefully on a red cell we can usually make out a fine wavy so-called molecular motion in it. This is quite different from the active amoeboid movement observed in dying cells, and from the rapid dancing of malarial pigment."

In modern textbooks of cytology and haematology no reference to this motion is found. On the contrary, the absence of structure as seen by all methods of microscopy except the polariscope is stressed. Yet motion is not possible in the absence of structure.

During the examination of avian and amphibian erythrocytes by phase contrast illumination a rapid and regular oscillatory movement was easily noted in the cytoplasm. The cytoplasm of normal and abnormal erythrocytes revealed the same motion, and indeed all erythrocytes examined, including those of goose, chicken embryo, hen, rabbit, guinea-pig, and mouse, show it clearly. It is best seen in the large nucleated erythrocytes of birds and frogs.

" Brownian movement " is the oscillation of visible particulate material when suspended in fluid. The vibratory phenomenon here discussed does not involve motion in particulate material within the limits of resolution of the microscope. No such particulate material exists in the erythrocyte. The phenomenon consists of a rapidly alternating change of the refractive index of the cytoplasm, leading to an appearance of alternate lightening and darkening of certain areas. These areas are, roughly speaking, arranged radially, and are exceedingly close together, so that the whole of the content gives the appearance of intense and continuous agitation.

The cell membrane of mammalian erythrocytes, when examined under a cover-slip pressed firmly down, shows a continuous irregular deformation of outline, which can be clearly seen by dark ground illumination ; by careful focusing it can be noted that the upper surface, as well as the edge, is in continuous motion. No such motion can be seen with the erythrocytes of bird or frog ; the latter indeed have, in the cases examined, on their surface minute granules which remain motionless. It follows therefore that the apparent vibration in the cytoplasm is not due to a change in refractive index of the contents as the cell varies locally in thickness owing to movement of the cell membrane, but to a periodic change of some kind in the cell contents. As will be related, this vibration can be inhibited by sodium fluoride.

Since the geometric form of erythrocytes is conditioned by the physical nature of their environment, it is necessary to adopt a special technique to observe the vibratory motion. In saline or in Tyrode's solution mammalian erythrocytes assume a spherical form ; this form is also assumed with many reagents. Blood must therefore be collected in minimal amounts of 3% sodium citrate, or examined fresh and undiluted.

When crenated, erythrocytes show poor vibration ; when spherical, they show none, and indeed they appear to be dense and compact. The best condition for viewing cells is obtained by placing a small drop on a slide, and pressing the cover-slip very firmly down under blotting-paper.

When examined by normal transmitted light, vibratory movement can sometimes be seen if the iris diaphragm is well stopped down, but it is most difficult to observe. By dark ground illumination it is very clearly seen in avian cells, but in mammalian cells it is in part obscured by the motion of the cell membrane. It is best observed by phase contrast. The illuminant for phase contrast microscopy is most important, especially with high power objectives. After experimenting with many types, the best was found to be the B.T.H. Mazda

erythrocytes show no trace of vibration, but they do frequently contain minute granules in Brownian movement.

Vibration in Other Cells.—Nothing resembling this has been noted during long examination of other mammalian cells, or in the cells of lower forms of life—e.g., *Daphnia*. On a warm stage the granules in many cells oscillate, but this appearance is of an entirely different order from the comprehensive erythrocytic vibration.

Reticulum in unstained reticulocytes cannot be resolved by phase contrast, but blood with 30% of reticulocytes showed vibration in all the erythrocytes.

FIG. 1.—Avian erythrocytes in plasma. Phase contrast × 900.

FIG. 2.—Avian erythrocytes in plasma with 0·1 ml. M sodium fluoride added per ml. plasma. Phase contrast × 900.

mercury vapour electric discharge lamp, box type ME 250 W/50/5, as recommended by Clegg and Foster-Carter (1946).

Experiments

Duration and Period of Vibration.—Vibration continues unaltered up to 48 hours in many cases. In general it appears to persist until haemolysis sets in. The oscillatory movement appears to be of the same periodic order as that of minute particles suspended in the plasma. Attempts to measure the period with a stroboscope were not successful.

Temperature.—No change in vibration was observed between 0° C. and 37° C. When further warmed, haemolysis sets in, and vibration ceases to be observable.

Haemolysis.—In whatever way haemolysis is produced—i.e., by the action of complement on sensitized cells—vibration gradually ceases. " Ghost "

When vitally stained by cresyl blue the reticulocytes are identifiable ; the reticulum remains motionless but the cytoplasmic vibration continues.

The abnormally shaped erythrocytes seen in anaemia also show vibration. It is visible in erythroblasts but not in pro-erythroblasts either from bone marrow or in the peripheral circulation. Not until a halo is visible around the nucleus by phase contrast is vibration visible ; this halo is not seen in pro-erythroblasts or in more primitive cells of the series.

Effect of Gases.—Vibration continues in cells which have been exposed to an atmosphere of pure oxygen, nitrogen, carbon dioxide, and carbon monoxide.

Effect of Certain Chemical Reagents.—Vibration ceases immediately with formalin at a concentration of 1 in 2,000 of the 40% solution.

The effect of sodium fluoride is noteworthy. When 0.1 ml. of a molar solution is added to 0.9 ml. of citrated blood, there is no immediate change in the appearances, but within three minutes vibration ceases

with mammalian erythrocytes. Avian erythrocytes also cease to show vibration, but in addition show another change. By phase contrast there is normally no halo around the nucleus, but after the addition of sodium fluoride a marked halo develops, and the cytoplasmic contents appear to be withdrawn and condensed towards the periphery. If avian cells treated with sodium fluoride are fixed and stained by Leishman's stain, they are, however, indistinguishable from normal cells.

Discussion

The first observers of this motion accepted it as "molecular movement," and apparently saw it by transmitted light. They worked in an era of scrupulous microscopical observation of living cells ; to-day we are rather limited by the artifacts of fixation and staining. It certainly is not an artifact produced by the alternation of current to an A.C. lamp, since it is equally well seen with a D.C. arc. A reason has been given for excluding the suggestion that the apparent motion is due to oscillations in the cell membrane ; indeed by phase contrast the impression given is definitely one of turbulence in the cytoplasm.

Solutions of haemoglobin in distilled water or in gelatin show no trace of the motion, and ghost erythrocytes equally show no motion. Either the haemoglobin when inside the cell itself occasions the motion, or it acts as an indicator, making motion visible. This might, for example, be the case if it were periodically subject to focal concentration, with a change of refractive index. It is not possible to resolve any particulate matter in erythrocytes by any microscopical technique ; the haemoglobin molecule is well outside the limits of resolution.

The main function of haemoglobin is the acceptance and transference of oxygen. The motion, however, cannot be related to this, since when saturated with carbon monoxide the cell still vibrates. It also vibrates when sodium hydrosulphite is added, until haemolysis sets in.

When the cell is "fixed" by formalin vibration ceases, doubtless owing to physical alteration in the colloidal contents. The action of sodium fluoride is of greater interest, particularly since it

appears to produce an alteration of the distribution of haemoglobin in the cell, at least in avian erythrocytes.

Whereas many reagents added to suspensions of erythrocytes in plasma tend to produce changes in the geometrical form of erythrocytes, sodium fluoride in the strength used does not ; its effect is only noted optically by the cessation of vibratory motion in the mammalian erythrocyte, and redistribution of haemoglobin in the avian. Wilbrandt (1940) has shown that sodium fluoride causes permeability to cations in erythrocytes, and this is said to be related to interruption of glycolysis. The normal erythrocyte is impermeable to potassium ; this characteristic is altered by sodium fluoride. So far as the experiments related here give any indication of the nature of the vibratory motion, they suggest that it is related to glycolysis. The vibratory motion itself is an indication that the refractive index of the cytoplasmic contents is constantly changing ; the alternation of dark and light seen by phase contrast is probably due to changing concentrations of the haemoglobin, and this may be related to metabolism, and particularly to glycolysis.

Summary

Mammalian, avian, and amphibian erythrocytes show a vibratory motion in the cytoplasm, best seen by phase contrast. No other cell of many examined, whether from mammals or from lower forms of life, exhibits this motion. It is seen in reticulocytes and erythroblasts, but not in proerythroblasts. It is not seen in de-haemoglobinized erythrocytes or in a solution of haemoglobin. It is inhibited by sodium fluoride, which produces a redistribution of haemoglobin in avian erythrocytes. The possible relation to glycolytic metabolism is discussed.

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ISO-IMMUNIZATION BY RARE Rh-ANTIGENS AS A CAUSE OF HAEMOLYTIC DISEASE OF THE NEWBORN AND TRANSFUSION REACTIONS

BY

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It is well known that Rh antibodies develop in persons who are nearly always Rh-negative, either as a result of immunization during pregnancy, or as a result of repeated transfusions when Rh-positive cells are given to Rh-negative recipients.

During the last two and a half years we have had an opportunity to study 885 families in which the mothers were immunized by pregnancy. Nearly all these cases were caused by immunization to the D (Rh₀) antigen ; in some instances, however, antibodies against the C (rh′) or E (rh″) antigens were developed as well.

Of the 13 blood transfusion reactions due to Rh antagonism, all but two were similarly caused by the most important of the Rh antigens, the D or Rh₀ antigen, which was lacking in the recipient.

In the literature only a few cases are reported, in which Rh antigens other than D (Rh₀)—that is to say the C, E, c, d, or e antigens—have been found to be the only cause of Rh immunization. In this paper we propose to give details of the few exceptional cases due to immunization to these rarer antigens, and to mention at the same time the possibility of producing antibodies against them by artificial immunization.

Immunization Due to the E (rh″) Antigen

Case 1 (K.F.).—The first child, born in 1934, died seven weeks after birth from gastro-intestinal infection. In 1938 a full-term normal infant was delivered. This was followed by a pregnancy in 1946, which terminated with the birth of a seven-months stillborn foetus. In 1947 a full-term child was born. This child, however, died two days after delivery, without icterus but with symptoms of cerebral lesions (kernicterus ?).

Serological tests gave the following results:

Father's cells: O M P Rh-positive, subtype cDE (Rh₂).

Mother's cells: O N pp Rh-positive, subtype CDe (Rh₁).

Infant's cells: O MN Rh-positive, genotype CDe/cDE(Rh₁Rh₂).

In the mother's serum a pure antibody, type anti-E (anti-rh″), was present, of titre 1:8 in saline as well as in albumin. The same titre was found by means of the indirect Coombs test.

Case 2 (K.).—The first two children were healthy. The third child shortly after birth showed symptoms of a mild icterus, but developed quite normally. The fourth child was brought to hospital when six weeks old. The paediatrician (Bakx, 1949) found a hepatosplenomegaly. The haemoglobin was 43%. No nucleated red cells were found in the blood smear.

Although the mother was group O Rh-positive, the infant's group O Rh-positive red cells were strongly agglutinated by the serum of the mother.

Serological tests gave the following results:

Father's cells: A Rh-positive, CDe/cDE (Rh₁Rh₂).

Mother's cells: O Rh-positive, CDe/CDe (Rh₁Rh₁).

First child: A Rh-positive, CDe/CDe (Rh₁Rh₁).

Second child: O Rh-positive, CDe/cDE (Rh₁Rh₂).

Third child: O Rh-positive, CDe/cDE (Rh₁Rh₂).

Affected baby: O Rh-positive, CDe/cDE (Rh₁Rh₂).

In the maternal serum a pure antibody of the type anti-E (anti-rh″) was present: titre in saline 1:16: titre in albumin 1:64.

Cases similar to the two above-mentioned examples have been described by Rice and Watson (1948), Race (1946), and Dick (1947).

Clearly iso-immunization during pregnancy by the E (rh″) antigen alone is very exceptional.

Immunization Due to the C (rh′) Antigen

Case 3 (B.L.).—The first child, born in January, 1943, was normal, but a second pregnancy terminated with the birth of a full-term, stillborn child in July, 1945. A third pregnancy ended with the birth of a six-months hydropic foetus in June, 1946. In August,

1947, a baby was born with mild symptoms of erythroblastosis foetalis. This child was treated by exchange transfusion and developed quite normally, although jaundice persisted for several weeks.

Serological tests:

> Father's cells: O Rh-positive, CDe/Cde (Rh₁rh').
> Mother's cells: A Rh-negative, cde/cde (rhrh).
> First child: A Rh-positive, CDe/cde (Rh₁rh).
> Affected infant: O Rh-positive, Cde/cde (rh'rh).

By repeated examinations it was confirmed that the child's cells belonged to the genotype Cde/cde (rh'rh), and that the absence of reaction with anti-D serum was not due to the presence of a weak D or Dᵘ antigen, which might have been overlooked early in life. In the mother's serum anti-C was present at a titre of 1:8, and anti-D at 1:32; both antibodies were of the incomplete type. The presence of the anti-D antibody may be explained by previous immunizations with D antigen. There is no direct proof that the difference in clinical picture between the last-born infant (only mildly affected) and the previous children (severely affected) was the result of a difference in the titre of antibodies, or due to the fact that the child possessed only antigen C. It must be added that we have never found a direct relation between the type or titre of antibodies and the clinical picture, although in general a high or increasing titre of incomplete antibodies, type anti-D, is often associated with a severe form of the disease.

As far as we know, only a few examples of erythroblastosis foetalis due to the presence of the antigen C (rh') alone have been described. Waller, Levine, and Garrow (1944) found a case of erythroblastosis foetalis, in which the mother's red cells belonged to group A₁, cDE (Rh₂), the infant's red cells to A₁, CDe (Rh₁), and an antibody of the type anti-C (rh') was found in the maternal serum. Two other cases are reported by Tisdale (1949) and by Drummond (1948).

Immunization Due to the Cᵂ (rh″ ′) Antigen

Erythroblastosis foetalis due to the allelomorph Cᵂ (rh″ ′) is exceedingly rare. The antigen Cᵂ was discovered by Callender and Race in 1946, and the first case of Cᵂ–C antagonism causing erythroblastosis foetalis was described by Lawler and van Loghem (1947).

The infant's red cells belonged to the genotype CᵂDe/CDe (Rh₁ ᵂRh₁), and the mother's cells to the genotype CDe/cde (Rh₁rh). In the maternal serum a pure anti-Cᵂ was present with a titre in saline of 1:2, and 1:128 in albumin, as well as by means of the indirect Coombs test. A second case of haemolytic disease of the newborn caused by Cᵂ–C antagonism has been reported by Broman (1948).

The rare occurrence of erythroblastosis foetalis as a result of iso-immunization by Cᵂ–C antagonism may be explained by the low frequency of this antigen, 2.5% in England and 4.2% in Holland.

Immunization Due to the c (hr′) Antigen

The antigen c (hr′) is responsible for rather a large number of cases of haemolytic disease of the newborn. The corresponding antibody anti-c (anti-hr′) was discovered almost simultaneously in 1943 by Levine and Race and Taylor in Rh-positive women.

In our material we have found four cases of erythroblastosis foetalis due to iso-immunization in pregnancy to the antigen c (hr′). The genotypes of the father's cells were cde/cde (rhrh), CDe/cde (Rh₁rh), or cDE/cde (Rh₂rh), and that of the mother's cells CDe/CDe (Rh₁ Rh₁). Pure antibodies of type anti-c (anti-hr′) in the complete and/or incomplete form were present in the maternal sera.

The sera were also tested with Cde/Cde (rh′ rh′) cells in saline, albumin, and with the indirect Coombs test, but an antibody of type anti-d was never found. The antigenic power of the d-antigen must be very weak, for there is an equal chance of producing anti-d as well as anti-c.

Brief histories of these pregnancies follow.

Case 5 (H.).—The first case in Holland was serologically examined and described in detail by Van Bolhuis (1948).

Out of this marriage of father, group O cDE/cde (Rh₂rh), and mother, group A CDe/CDe (Rh₁Rh₁), 12 children were born. All of them developed jaundice shortly after birth, and three of them died, the third child from bleeding from the umbilical cord and the seventh child half an hour after birth, with jaundice and ascites. The ninth pregnancy ended with the birth of twins; one of them was very jaundiced and survived only two days, the other one was more mildly affected, although with a strong erythroblastaemia, and developed quite normally. The tenth child was also only mildly affected, but the eleventh now has the sequelae of kernicterus. The last child, born in 1948, was treated with exchange transfusion and developed quite normally.

In the maternal serum an antibody type anti-c (anti-hr′) was found at a titre of 1:64 in saline and 1:128 in albumin.

Case 6 (v.S.).—In this family, after two normal children in 1939 and 1944, an infant was born with symptoms of a congenital haemolytic anaemia. The haemoglobin level was 40% and erythrocytes 1,860,000 per c.mm. There was no jaundice and no hepatosplenomegaly.

> Father's cells : O cde/cde (rhrh).
> Mother's cells : O CDe/CDe (Rh₁Rh₁).
> Infant's cells : O CDe/cde (Rh₁rh).

In the maternal serum an antibody type anti-c (anti-hr′) was present, at a titre of 1 : 16, in the incomplete form only.

Case 7 (D.).—The history of this patient's pregnancies is marked by a number of stillbirths, with pregnancies terminating in the fifth, third, fifth, sixth, sixth, and sixth months. A living child was never born.

> Father's cells : A cde/cde (rhrh).
> Mother's cells : O CDe/CDe (Rh₁Rh₁).

An incomplete antibody type anti-c was present: titre in albumin 1 : 64; in saline, negative.

Case 8 (V.).—The first pregnancy ended in 1942 in a stillbirth, the second resulted in the birth of a normal child in 1943. In 1944 a premature stillborn child was born, 5½ months old. A child born in 1947 survived, but was jaundiced after birth. In 1948 the last child was born three weeks before term. This infant was very jaundiced and showed symptoms of kernicterus. Unfortunately it was treated with Rh-negative (cde/cde) O blood. It survived, however, and three months after birth its blood was investigated. The mother was found to belong to group A CDe/CDe (Rh₁Rh₁), the father to group A CDe/cde (Rh₁rh), and the infant to group O Cde/cde (Rh₁rh). In the maternal serum an antibody type anti-c was found, titre in saline 1 : 2, in albumin 1 : 16.

As may be concluded from the cases already described and Case 9, the c-antigen is a strong antigen compared with C and E. The frequency of iso-immunization due to the c-antigen in haemolytic disease of the newborn forms a striking contrast to the infrequency of immunization by C and E.

Blood Transfusion Reactions Due to Differences in Rh Type

Blood transfusion reactions due to differences in Rh type between the recipient and the donor are rarely encountered. One of the most important reactions is due to transfusion of Rh-negative blood to homozygous Rh-positive recipients.

Wiener (1948) reported two cases of transfusion reactions due to the presence of the iso-antibodies anti-c (hr′). Other cases have been reported by Speck and Sonn (1945), Sussmann (1947), and by Callender and Racē (1946).

We have encountered one case of a haemolytic transfusion reaction due to immunization by this antigen in a woman who was previously immunized by pregnancy.

Case 9 (V. de W.).—This woman received a blood transfusion for anaemia six days after delivery of her ninth child. Her haemoglobin was 43%. When about 50 ml. of blood had been given a severe haemolytic reaction developed, and the transfusion was immediately stopped. The red cells of the donor were agglutinated by the serum of the patient after 15 minutes' incubation at 37°. Further investigations of this reaction showed:

> Patient: A CDe/CDe (Rh₁Rh₁).
> Donor: A CDe/cDE (Rh₁Rh₂).

In the serum an iso-antibody of the type anti-c (anti-hr′) was present, agglutinating in saline as well as in albumin (titre 1 : 512). The patient's husband belonged to group A cDE/cde (Rh₂rh), and four of the children to group O CDe/cde, A CDe/cde, O CDc/cde, and A CDe/cDE respectively.

It was remarkable that the history of pregnancies did not show striking evidence for erythroblastosis foetalis. Only one of the nine children, the eighth, born in 1945, was stillborn. The course of all the other pregnancies was quite normal. The children were delivered at full term and did not show any symptoms of haemolytic disease. Even the last child, born six days before the transfusion, was quite healthy without the slightest symptoms of anaemia or jaundice. Unfortunately it was impossible to perform haematological investigations shortly after birth, but undoubtedly the antibody anti-c must have been present in the maternal serum, and presumably in the blood of the last-born child without affecting it, although its blood group was A CDe/cDE. The woman had never been transfused before, neither had she received intramuscular injections of blood. All the children probably stimulated the production of anti-c (anti-hr′), for the antigen c in the blood cells of the father was present in the homozygous form, cDE/cde.

The antibody anti-d was detected by Diamond (1946) and later reported by Hill, Haberman, Everist, and Davenport (1948). It has never been encountered in the pure form, but only in combination with anti-c.

The antibody anti-e (anti-hr″) was first discovered by Mourant (1945) in a patient of the genotype cDE/cDE (Rh₂Rh₂) who had received multiple transfusions. Recently Wiener and Peters (1948) have described a second example resulting from immunization by blood transfusion, and Moullec (1948) has reported a third case in a 12-year-old girl, also due to multiple transfusions; in this case anti-e was found together with anti-C. There is no example of iso-immunization in pregnancy by this antibody.

We have never encountered the antibody type anti-d (anti-Hr₀) and anti-e (anti-hr″) either as a result of pregnancies or of transfusions. Transfusion reactions due to differences in subtypes of Rh-positive blood between donor and recipient are likewise exceedingly rare. The only instance we have encountered was of a patient of blood group A MN cDe (Rh₀). The scanty clinical

data available concerning this patient are presented below.

Case 10.—J., 23 years old, was wounded by bomb splinters in July, 1947, and received four transfusions without any reaction. The patient was treated in 1948 with blood transfusions and penicillin for subacute bacterial endocarditis which had developed as a result of local lesions in the heart caused by bomb splinters. Seven transfusions of a pint of blood were given in a period of about five weeks. The second transfusion was followed by chills, and so was the fourth transfusion. A rather severe febrile reaction also followed the last transfusion, but there was no positive evidence of a haemolytic reaction (no haemoglobinuria or jaundice).

Patient's blood cells: A MN cDe (Rh₀).

Donor's blood cells: A MN CDe/cDE (Rh₁Rh₂).

It will be seen from Table I that all the reactions were caused by cDE blood.

TABLE I

CASE 10: REACTIONS TO TRANSFUSION

Date	Transfusion No.	Reaction	Donor's Blood Group
July 1947	4 transfusions	No reaction	A CDe (Rh₁)
10-9-48	First	,, ,,	A cDE (Rh₂)
14-9-48	Second	Chill	
17-9-48	Third	No reaction	
21-9-48	Fourth	Chill	A cDE (Rh₂)
26-9-48	Fifth	No reaction	
6-10-48	Sixth	,, ,,	A CDe (Rh₁)
21-10-48	Seventh	Chill	A cDE (Rh₂)

After the last transfusion a pure antibody type anti-E (anti-rh″) was present in the patient's serum, only reacting in albumin at a titre of 1 : 16.

Race (1946) has found several cases of transfusion reactions due to the E antigen.

TABLE II

EXPERIMENTAL FORMATION OF PURE ANTIBODY TYPE ANTI-Cʷ

Date of Injection and Serial No.		Amount of O Cʷ Cells Injected (ml.)	Titres of Antibodies (Anti-Cʷ)	
			Saline	Albumin
26.4.48	1st			
5.8.48	21st	0·5		1 : 8
16.8.48	22nd	0·5		1 : 8
19.8.48	23rd	1·0*	1 : 2	1 : 16
23.8.48	24th	1·0	1 : 16	1 : 64
25.8.48			1 : 32	1 : 128

* 0·5 ml. of typhoid vaccine (triplovaccin diluted 1 : 3) given intramuscularly.

Antibody Formation Due to Artificial Immunization

In previous articles we reported the production of the rare antibodies anti-C and anti-E as a result of frequent immunization with small quantities of Cde and cdE cells in Rh-negative volunteer donors. The influence of heterospecific immunization on the production of antibodies against the injected antigen C and E was described. By this means we succeeded in producing two sera with anti-C and one with anti-E, the last one without the injection of heterospecific antigens.

After this method was proved to be successful we tried to make the rare antibody anti-Cʷ in the same way. Three of our volunteer donors were injected with cells of the Rh group CʷDe/cde. Injections of 0.5 ml. of a 50% suspension of these cells were given twice a week. The Rh groups of the donors were CDe/cde. As will be seen from Table II one of the donors (Group O MN CDe/cde) reacted with the formation of a pure antibody type anti-Cʷ. It is highly probable that the titre 1 : 8 found after the twenty-first and twenty-second injections was increased by the administration of typhoid vaccine injected intramuscularly. After 24 injections the titre in saline was 1 : 32 and in albumin 1 : 128. At this time the donor was bled and the immunization procedure stopped.

Summary

In 885 cases of Rh iso-immunization during pregnancy, the antigen D was almost invariably concerned. In these cases the mother was D-negative and the infant D-positive, and the mother formed anti-D, with or without anti-C or anti-E. In one case an infant of genotype Cde/cde was affected by anti-C in its mother's serum.

In eight exceptional cases the mother was D-positive and formed anti-c (4 cases), anti-E (2 cases), or anti-Cʷ (1 case).

Similarly, of 13 blood transfusion reactions due to Rh incompatibility, all but two were caused by the formation of anti-D by a D-negative recipient. In the two exceptional cases, anti-c and anti-E were concerned.

The rare antibody anti-Cʷ was produced by one out of three volunteers who were given repeated intravenous injections of Cʷ blood.

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TECHNICAL METHODS

A SLIDE METHOD FOR DEMONSTRATING SOLUBLE HAEMOLYSIN

BY

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The following method of testing the haemolytic streptococcus for soluble haemolysin has been found to give good results, and no indefinite results (as are sometimes found by the tube method) have been encountered. The main advantage of the method is that positive results are easily read and are not screened by undissolved red cells, as occasionally happens in the tube method, thus making the readings difficult.

Method

The streptococcus to be tested is subcultured in 20% serum broth and incubated overnight. A conveniently sized loopful of the culture is placed on a $\frac{7}{8}$ in. square (No. 1) coverslip and mixed with a loopful of normal horse cells.

The mixture is mounted by touching the drop with a slide as in preparing ordinary wet film. Finally the coverslip is sealed all round with vaseline and incubated at 37° C. A positive result is indicated by a complete transparency of amber colour in about three hours as a rule, whereas in a negative result the preparation remains opaque (see Fig.). Late afternoon preparations may be incubated overnight. The results are judged simply by holding up the slide to the light.

The best results are obtained with a proportion of two or three volumes of cells to one volume of culture, but exact amounts are not essential. As a larger volume of cells than of the broth culture is normally picked up by the loop, one loopful of each works well in practice. (The normal horse blood is undiluted, that is, as it is used for adding to nutrient agar for making blood-agar plates.)

SEALING TOOL FOR WET PREPARATIONS.—A quick and effective tool for sealing off wet preparations was devised in the following manner.

A piece of soft metal about $\frac{1}{4}$ in. wide and $3\frac{1}{2}$ in. long by 1/32 in. thick was obtained and bent with pliers into a square with sides approximately $\frac{3}{4}$ in. (For the metal strip a faeces spoon was used.) A metal swab stick was suitably bent and a handle soldered on vertically: or holes could be drilled and the ends of the swab stick bent and inserted. To use the device it is only necessary to hold the handle and heat the square of metal in a bunsen flame for a few moments. Then the bottom edge of the heated metal is pushed into a petri dish of vaseline, removed, and held in such a way as to touch or almost touch the perimeter of the coverslip. The melted vaseline then runs on to the glass and forms an effective and neat seal.
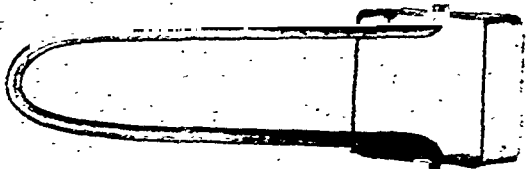
Photograph of slide showing negative (left) and positive results, and (below) the tool described in the text.

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THE DETERMINATION OF SERUM IRON WITH FERRICYANIDE

BY

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There are numerous reagents which produce a coloured complex with iron and are, in general, suitable for the colorimetric determination of this substance. The minute amount of iron present in serum, however, restricts determinations to the iron thiocyanate or o-phenanthroline reactions.

In the thiocyanate method the colour fades rapidly and it is necessary to extract the coloured ferric thiocyanate complex with an organic solvent, in which many of the salts which inhibit the colour development and otherwise interfere are insoluble. The o-phenanthroline method is less sensitive than the thiocyanate method, but has the advantage that the colour developed is stable for long periods. The present work was undertaken to develop a method in which the colour was specific, stable, and intense enough to allow good readings to be obtained in the photoelectric colorimeter at the low level of iron concentration encountered in serum.

Ferrous iron forms a blue colour with potassium ferricyanide, and it was decided to utilize this colour for the iron determination. The non-haemin iron is liberated from the serum by incubation with hydrochloric acid, the proteins precipitated with trichloracetic acid, and the iron in the protein-free solution reduced to the ferrous state with hydrazine sulphate, as described by Barkan and Walker (1940). Potassium ferricyanide is then added and the blue colour measured on the Spekker photoelectric absorptiometer.

Experimental Work

The coloured iron ferricyanide complex is soluble in excess of the reagent, and the present investigation shows that the colour can be used for the quantitative determination of iron in serum. The blue colour, combined with the yellow of the excess ferricyanide, gives a green colour, the blue component of which is proportional to the iron concentration.

If potassium ferricyanide and hydrazine sulphate are left together in an acid solution, the solution after three or four hours assumes a greenish tinge which gradually increases in intensity. This colour, which is due to the decomposition of the ferricyanide, bears no relationship to the iron concentration of the test solution. In an actual iron estimation the colour due to the iron reaches a maximum in a quarter of an hour, is stable for a further two hours, and then begins to increase in intensity as described above.

Calcium, lead, and arsenic do not interfere with colour development at all. In the amounts normally present in serum, zinc and copper do not interfere, but when present in high concentrations, cause precipitation of their respective ferricyanide complexes. Oxalate completely inhibits colour development, and for this reason serum, and not plasma, was used throughout our experiments. Variation in the concentration of potassium ferricyanide used causes no change in the final reading obtained, and practically no difference was found when the concentration used differed as much as from 5% to 20%. Slight variation in the concentration of hydrazine sulphate causes no appreciable difference.

Provided the hydrochloric acid used to liberate the non-haemin iron does not exceed a concentration of 10% (volume/volume), the colour will not be affected, but should it rise above this, partial fading will take place, until at a concentration of 20% (v/v) no colour develops at all. The final solution, therefore, must not be stronger than 0.2N in respect of hydrochloric acid. Similarly, the amount of trichloracetic acid used to precipitate the proteins must not exceed the 2 ml. of 20% (weight/volume) otherwise fading will take place. Under the conditions described in our method, there is no interference from the hydrochloric acid or the trichloracetic acid. Sulphuric acid does affect the colour, but only when the solution is greater than 0.1N in respect of sulphuric acid. Phosphate, sulphate, and chloride do not interfere.

Method

The following is a detailed description of our method.

Reagents.—The following reagents are used:

1. Hydrochloric acid, 5% (v/v).

2. Trichloracetic acid, 20% (w/v).

3. Hydrazine sulphate. 1% (w/v), freshly prepared before use.

4. Potassium ferricyanide, 10% (w/v).

Double-distilled water is used in making up all solutions, which are stored in acid-washed bottles. All other glassware used is also acid-washed, and then washed acid-free with double distilled water.

Procedure.—To 4 ml. serum in a thick walled test tube 2 ml. 5% hydrochloric acid is added and incubated at 37° C. for one hour. This is cooled to room temperature and 2 ml. 20% trichloracetic acid added. It is then mixed, allowed to stand for at least one hour, and centrifuged for 15 minutes at 2,500 revolutions per minute. It is important that the mixture be allowed to stand for at least one hour, otherwise a clear supernatant fluid will not be obtained. If possible it should be left overnight before centrifuging.

To another test tube 4 ml. of the supernatant fluid are transferred. A blank is prepared in a third test tube by taking 2 ml. water, 1 ml. 5% hydrochloric acid, and 1 ml. 20% trichloracetic acid. To both 1 ml. 1% hydrazine sulphate is added, shaken, and left for five minutes. To each is added 1 ml. 10% potassium ferricyanide and 4 ml. water. The test tubes were left to stand for 15 minutes to allow the colour to develop. The colour is then measured in the Spekker photoelectric absorptiometer, using 1 cm. cell and the Spekker red filter OR2. (We have found it convenient to set the Spekker drum at 0.200 using the blank in the solvent cell. The instrument is then adjusted until there is no deflexion in the galvanometer, and is ready for taking a series of readings. The unknown solution is placed in the other cell, and the drum rotated until there is no deflexion. The reading will be less than 0.200. It has been found that this method saves considerable time when there are a number of estimations.)

To draw up a reference curve for the Spekker absorptiometer, we took tubes containing 0, 2.5, 5.0, 7.5, 10.0 µg. iron in 4 ml. water, added 1 ml. 1% hydrazine sulphate, 1 ml. 10% potassium ferricyanide, and 4 ml. water. The reference curve is read in the Spekker absorptiometer after 15 minutes.

Results

Recoveries of inorganic iron added to serum were shown to be quantitative, and from the figures shown on Table I will be seen to compare favourably with those obtained by existing methods.

A number of iron determinations were carried out on serum from patients with different clinical conditions, and the results are shown to lie between the usual limits of 100 and 200 µg. per 100 ml. (Table II).

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TABLE I
RECOVERY OF INORGANIC IRON ADDED TO SERUM

No.	Initial Iron Content (µg. Fe)	Iron Added (µg. Fe)	Total Iron Determination (µg. Fe)	Iron Recovered (µg. Fe)	Recovery (%)
1	1.22	2.50	3.75	2.53	101.2
2	1.50	4.00	5.35	3.85	96.25
3	1.65	2.50	4.14	2.49	99.6
4	1.83	2.50	4.23	2.40	96.0
5	2.10	5.00	6.90	4.80	96.0
6	1.79	2.50	4.30	2.52	100.4
7	1.74	5.00	6.48	4.74	94.8
8	1.12	2.50	3.53	2.41	96.4
9	2.80	5.00	7.50	4.70	94.0

TABLE II
THE IRON CONTENT OF HUMAN SERUM

No.	Clinical Condition	Serum Iron (µg. Fe/100 ml.)
1	Infective hepatitis	140
2	Epistaxis	90
3	Sciatica	125
4	Tabes dorsalis	175
5	Obstructive jaundice	85
6	Hepatic cirrhosis	115
7	Hepatitis	165
8	Duodenal ulcer	170
9	Splenic anaemia	80
10	Pernicious anaemia	125
11	Pernicious anaemia	430
12	Pernicious anaemia	430
13	Haemolytic anaemia	115
14	Lead poisoning	225
15	Lead poisoning	160
16	Lead poisoning	145
17	Lead poisoning	190
18	Lead poisoning	190
19	Lead poisoning	125
20	Lead poisoning	240
21	Lead poisoning	205

Summary

A method for the determination of serum iron is described. The advantages of the method lie in the facts that only readily available chemicals are required, there is very little interference by other substances, and the colour, which is reasonably stable, develops rapidly.

REFERENCE

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ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections · bacteriology ; biochemistry ; haematology ; and morbid anatomy and histology.*

BACTERIOLOGY

Antibody Formation in Early Infancy Against Diphtheria and Tetanus Toxoids. COOKE, J. V. (1948). *J. Pediat.,* 33, 141.

The hypothesis that production of antibodies in new-born infants is relatively inefficient has been re-examined in the light of the desirability of early immunization against whooping cough, concomitant immunization against diphtheria, and the known satisfactory response in infancy to smallpox immunization.

A group of children aged from 1 month to 14 months, of whom the majority were under 6 months of age, was treated by subcutaneous injection of combined diphtheria and tetanus toxoid after a previous titration for anti-bodies. A second injection was given 2 months later and after a further month a blood sample was re-titrated. Results of titrations are given.

It appears that even small amounts of antibodies may prevent immunity being produced. The percentage of passively immune children in the early months of life revealed in this study was lower than that reported by previous workers. This appears to be due to the diminished prevalence of diphtheria, and number of Schick-negative adults. It may be concluded that at the present time in the United States one-third to one-half of all infants will be passively immune in the early months of life. It would therefore appear advisable to defer diphtheria immunization until after the sixth month. Since, however, evidence of antibody production appears to be satisfactory, even in the newborn, and in view of the high mortality rate of pertussis in the first months, it is advisable that pertussis vaccination be carried out in the sixth, ninth, and twelfth weeks and diphtheria (and, if desired, tetanus) toxoid be given in the seventh and ninth months. *G. T. L. Archer.*

The Use of Combined Antigens in the Immunization of Infants. FLEMING, D. S., GREENBERG, L., and BEITH, E. M. (1948). *Canad. med. Ass. J.,* 59, 101.

Some 200 children (average age about 4 months) were divided into groups and immunized with diphtheria toxoid, diphtheria toxoid and pertussis prophylactic, or with diphtheria toxoid plus pertussis prophylactic plus tetanus toxoid. Two doses of material were given at intervals of 3 weeks, and the immunity response was estimated 3 weeks after the second injection: to diphtheria prophylactic by estimation of serum antitoxin by rabbit skin testing; to *Haemophilus pertussis* by slide or tube agglutination tests with serum; and to tetanus by serum antitoxin tests in guinea-pigs. Before immunization no child possessed agglutinins against *H. pertussis;* 33% had at least 0.004 unit diphtheria antitoxin; 6% had tetanus antitoxin. The results below are based on those children without detectable antitoxin.

The response to diphtheria toxoid is considerably increased when it is given in combination with pertussis prophylactic and tetanus toxoid. The response to pertussis prophylactics is substantially unaltered by addition of other prophylactics. The presence of tetanus toxoid in the mixtures led to the production of an average of 0.021 unit tetanus antitoxin (1 ml. prophylactic mixture given). Reactions were not increased in severity by combination of prophylactics. On these grounds it is claimed that combined prophylactics are effective and desirable. *C. L. Oakley.*

Penicillin in Diphtheria. Report of a Sub-committee of the Public Health Laboratory Service. CRUICKSHANK, R., GILLESPIE, E. H., GOLDIE, W., HARRIS, H. J., JEBB, W. H. H., KING, G. J. G., MARTIN, P. H., PARKER, M. T., and MACDONALD, A. (1948). *Lancet,* 2, 517.

The penicillin sensitivity of 284 strains of *Corynebacterium diphtheriae*, isolated in different parts of Britain, was tested (technical details given), and an attempt was also made to find out whether penicillin treatment shortened the time of carriage of the organism in acute cases of faucial diphtheria or aided its elimination from persistent throat carriers. The clinical effect of penicillin on diphtheria was not considered.

The results showed that gravis strains of *C. diphtheriae* are rather more resistant than intermedius strains, and intermedius strains more so than mitis strains. Most of the strains tested required two or three times as much penicillin for *in vitro* inhibition as did the standard strain of staphylococcus, which was inhibited by 0.1 unit of penicillin per ml. of medium.

In treating acute cases of diphtheria antitoxin was used as well as the following doses of penicillin : adults and children over 10 years, 60,000 units 4-hourly during the day only (240,000 units daily) ; children 5 to 10 years old, two-thirds of the above ; children under 5 years, one-third of the above. Of 65 cases thus treated, 26 of 37 receiving a three-day course and 23 of 28 receiving a six-day course became free from the infecting organism within 4 days of the end of treatment. This compared favourably with previous rates of clearance published for cases treated with antitoxin only, and also with results in a small control group of 22 cases, noted by the present authors, in which only 3 had the first of a series of consecutively negative swabs within a fortnight of admission to hospital.

The authors consider that it may be easier to prevent the appearance of a convalescent carrier than to cure the established carrier condition, and they advocate that penicillin therapy should be begun as early in the disease as possible.

The Effect of Wetting Agents on the Growth of Tubercle Bacilli. DUBOS, R. J., and MIDDLEBROOK, G. (1948). *J. exp. Med.,* 88, 81.

It has been shown that it is possible to obtain finely dispersed growth of tubercle bacilli by adding to the

medium certain non-ionic wetting agents. "Tween 80," a polyoxyethylene ester of sorbitan mono-oleate, has been most extensively used for this purpose.

A defect of tween 80 and of some other kindred substances is the fact that they are liable to enzymatic hydrolysis by lipases, which prohibits their use in media containing animal tissues, or fluids rich in these enzymes. This paper describes the study of another type of wetting agent—an arylalkyl polyether of phenol, known as "triton A 20"—which appears resistant to the known enzymes of animal tissues. This was demonstrated by growing strains of *Mycobacterium tuberculosis*, virulent and avirulent, in a basal synthetic medium containing serum, to which tween 80 and triton A 20 were added. Tween 80 lost its ability to disperse cultures in such media; triton A 20 did not.

Other differences were found between the actions of the two substances. Tween 80 increases yield of growth probably by supplying oleic acid to the bacilli. Triton A 20 does not, although it was found that growth can be enhanced in its presence when long-chain fatty acids or sphingomyelin are added to the medium. In concentration sufficient to cause dispersed growth, tween 80 (purified by removal of unesterified fatty acid) was innocuous to both virulent and avirulent strains. Triton A 20, while not affecting virulent strains, in similar concentration showed a marked toxic effect on the avirulent variants tested. Triton A 20 does not produce such a finely dispersed growth, in the case of virulent strains, as does tween 80. This, according to the authors, is due to a peculiarity of the morphology of virulent strains, which produce, in addition to amorphous bacillary clumps, an end-to-end growth of bacilli resulting in serpentine strands. This formation of long bacillary strands is suppressed by tween 80, but not by triton A 20. *T. D. M. Martin.*

Clinical Experiences with Tb 698 Treatment in Pulmonary Tuberculosis. (Kliniska erfarenheter vid behandlingen av lungtuberkulos med Tb 698.) KUHLMANN, F. (1948). *Nord. Med.*, 39, 1325.

This article is a report of the author's experiences in treating 66 cases, mostly of very advanced pulmonary tuberculosis, with the new drug "Tb 1/698/E" (a mixture of equal parts of sulphathiazole and a semicarbazone). In this small hospital the usual mortality rate was 7 to 8% per month. Patients were chosen in whom the condition was deteriorating and was almost certain to continue to do so. Any improvement was attributed to the drug, the condition being assessed on the weight, erythrocyte sedimentation rate, and results of clinical, radiological, and sputum examinations. The drug was given in doses of 250 mg. a day for 8 days and then twice a day for 7 weeks; after a month's rest the course was repeated. No serious complications occurred, but gastro-intestinal upsets were common at the beginning of the course and diabetics were found to need much more insulin. Normal patients had rather low blood-sugar levels. The results of treatment were encouraging and the hospital's mortality rate fell to 1 to 2% per month. Of 17 cases not considered severe the majority improved. In 34 of 49 severe cases the condition improved and only in 4 did it deteriorate further; 29 patients put on weight; the sedimentation rate fell in 39 (to normal in 4 cases), but rose again in 15. There was an increase in the lymphocyte count in 37 and an eosinophilia of over 4% in 25. Sputum generally became less and changed from positive to negative in 17 cases; the reverse happened in 2 cases. In only 8 patients was there any radiological improvement, but many of those with laryngitis were much improved.

Clinical and Epidemiological Significance of Scarcity of Tubercle Bacilli in Sputum. BERLIN, I. I., BERGMAN, S. M., IOSELEVICH, V. S., ROZANOVA, M. D., MELESHKEVICH, M. P., SABSHINA, E. Y., and NILOVA, E. M. (1948). *Probl. Tuberk.*, 3, 19.

Clinical observations, extending over a period of many years, on in-patients and out-patients showed that the finding of a few tubercle bacilli in the stomach washings or the sputum (especially on examination by the flotation method) is of great significance in making a correct differential diagnosis or ascertaining the presence of concomitant tuberculosis in a primary non-specific pulmonary condition. The history and other relevant data in 108 carefully selected cases (out of 200), under observation for a long time, are presented and tabulated. The evidence showed that examination of stomach washings by the flotation method is of the utmost importance in early diagnosis of exacerbations of a tuberculous process. The relation between the radiological picture and allergic reaction on the one hand and scarcity of *Mycobacterium tuberculosis* in the sputum and stomach washings on the other is discussed in detail. *H. P. Fox.*

Determination of Streptomycin Sensitivity of Tubercle Bacilli by Use of Egg-yolk Agar Medium. KARLSON, A. G., and NEEDHAM, G. M. (1948). *Proc. Mayo Clin.*, 23, 401.

Preparation of Herrold's medium is first described in detail. Briefly, nutrient agar containing 2% of glycerin is first put up in 120-ml. lots. When the medium is to be prepared the glycerin agar is melted and cooled, and to each lot is added the requisite amount of streptomycin in 1 ml. of distilled water. Immediately after the streptomycin has been added the yolk of a fresh egg is poured directly into each lot, the bottle inverted several times to mix, and the mixture poured into tubes and slanted. Streptomycin concentrations used by the authors were 1, 10, 50, and 100 μg. per ml. of medium.

As regards the bacterial inoculum in the sensitivity test, the authors state that tests may be done by inoculating sputum or other material on the medium without making a preliminary isolation, provided that the specimen is heavily laden with tubercle bacilli and the bacilli are uniformly distributed in it. They add, however, that in their experience most specimens have too few tubercle bacilli to permit the direct test, and this is especially true of specimens from streptomycin-treated patients. In most cases, therefore, a preliminary isolation has to be made, and sensitivity tests must be done on pure cultures. Several methods are suggested. The technique is given.

The test is usually read after 14 days' incubation, and experiments show that the streptomycin in the medium remains stable throughout this time. Of 149 cultures from patients who had received no streptomycin, all were resistant to only 1 μg. or less of streptomycin per ml. of medium. It was found that 245 strains of tubercle bacilli from clinical material had the same sensitivity to streptomycin on the egg-yolk agar as in Proskauer and Beck's liquid medium. Among advantages claimed by the authors for the method are : ease of preparation of the medium ; greater ease in reading differences of growth in a solid medium ; the fact that cultures,

particularly those which have been stored for a long time, which fail to grow in a liquid medium may grow on the egg-yolk agar ; individual colonies may be selected from solid medium for further study.

Enhancement of Growth of a Strain of *M. tuberculosis* (var. *hominis*) by Streptomycin. SPENDLOVE, G. A., CUMMINGS, M. M., FACKLER, W. B., and MICHAEL, M. (1948). *Publ. Hlth. Rep., Wash.*, 63, 1177.

During the routine testing of a strain of *Mycobacterium tuberculosis* derived from the sputum of a patient with pulmonary tuberculosis who had been receiving streptomycin for 96 days, it was noted that growth was poor in the control tube while it was abundant in all tubes containing streptomycin in concentrations of 1, 5, 10, 100, and 1,000 μg. per ml. The phenomenon was noted in Dubos liquid medium and on Loewenstein–Jensen medium. After the second transfer of this strain on Dubos medium with graded amounts of streptomycin, the authors were successful in reduplicating their findings in 5 of 7 trials. The cause of the discrepancy is not understood, but two possibilities are suggested : (*a*) the culture is a mixture of strains, or (*b*) unknown variables such as differences in lots of culture media and streptomycin may have altered the finding. They conclude that this strain shows partial dependence on streptomycin for growth, and have isolated a second strain from the same patient, three months after conclusion of streptomycin treatment, which showed the same characteristic. Streptomycin-sensitivity of a culture isolated from this patient before streptomycin therapy was begun was not tested. *T. D. M. Martin.*

Stomatitis due to Streptomycin. Report of Three Cases. BEHAM, H., and PERR, H. (1948). *J. Amer. med. Ass.*, 138, 495.

Three cases are reported of patients suffering from tuberculosis who during treatment with streptomycin developed painful stomatitis which disappeared when the treatment was stopped. Two of the patients had pulmonary tuberculosis; in the third the disease led eventually to acid-fast infection of the lungs. Streptomycin was given in doses of either 1 or 2 g. daily, and in each case the stomatitis developed 4, 5, and 12 weeks respectively after the treatment was started. The clinical picture in the 3 cases was a painful erosive membranous stomatitis which involved the whole mucous membrane of the mouth as well as the under surface of the tongue. Treatment did not prevent the vesicles from eroding, but as soon as streptomycin was stopped an improvement took place, and the lesions disappeared within a fortnight. When treatment was started again a similar rash broke out. There were no other toxic reactions.

Results of Streptomycin Treatment of 19 Patients with *H. influenzae* Meningitis ; Comparison with Earlier Results. (Uitkomsten van de streptomycinebehandeling bij 19 lijders aan influenza-meningitis, vergeleken met vroegere uitkomsten.) BRUINS SLOT, W. J., HULST, L. A., and STENVERS, H. W. (1948). *Ned. Tijdschr. Geneesk.*, 92, 2973.

A comparison is made of the results of treatment by different (Netherlands) physicians of two groups of cases of *H. influenzae* meningitis, one (32 patients, including 21 under 2 years of age) with drugs other than streptomycin, and the other (19 patients, comprising 17 children under 2 years, a child of 4, and one adult) receiving streptomycin with or without other drugs. Of

the first group 4 recovered and of the second group 14. Of the fatal cases in the second group 2 ran a fulminating course with death within 3 days ; in one case necropsy revealed a subdural abscess ; one patient had been ill for 27 days before treatment started, and the fifth bacteriological examination showed a *Listeria* to be present as well. *K. Kraaijenbrink (Excerpta Medica).*

Serologic Findings in Patients with Primary Atypical Pneumonia. MORGAN, H. R., and FINLAND, M. (1948). *Amer. J. clin. Path.*, 18, 593.

The sera from 89 patients with clinical and radiological appearances characteristic of primary atypical pneumonia were investigated. At least 2 serial specimens were tested in each case. Cold haemagglutination tests were considered positive when titres greater than 1 in 20 were obtained, and streptococcus MG agglutinin titres were considered significant when greater than 1 in 10. Complement-fixation tests for psittacosis and for meningo-pneumonitis were also carried out, a fourfold rise in titre or a titre of 1 in 16 with a subsequent rise being taken as diagnostic.

The results showed that about 80% of the patients developed cold haemagglutinins and agglutinins for the MG streptococcus. Only 4 out of 79 patients showed significant titres with psittacosis and meningo-pneumonitis antigens. Of these 4 patients, the sera of 3 were tested for cold agglutinins ; only 1 was positive. Five patients with erythema multiforme associated with atypical pneumonia all had positive cold agglutinin tests, while in 2 of them there was also serological evidence of infection with psittacosis virus. A history of recent close contact with birds was obtained in 10 of the patients with atypical pneumonia, though none of these had unequivocal evidence of infection with psittacosis virus. No evidence of Q fever was found in the case of 25 patients whose sera were tested for the appropriate antibodies. *R. B. Lucas.*

Disinfection of Tuberculous Sputum and Linen with Activated Chloramine Solutions. ARKHIPOVA, O. P. (1948). *Probl. Tuberk.*, 2, 50.

Experiments on disinfection were made with pure cultures of *Mycobacterium tuberculosis* and with infected sputum and linen. The bactericidal power of aqueous solutions of chloramine was enhanced by adding to them an equal amount by weight of ammonium chloride, sulphate, or nitrate. Pure cultures of *Myco. tuberculosis* were killed in the activated 0.05% and 0.1% solutions of chloramine in 2 hours and 1 hour respectively ; in the non-activated 1% solution the organisms survived for over 2 hours. The following solutions were suitable for effective disinfection of the collective samples of tuberculous sputum : a 2% solution of chloramine with 2% of activator (exposure for 4 hours in the ratio 2 parts of the solution to 1 part of the sputum) and 2.5% of activated chloramine (exposure for 2 hours in the same ratio). The activated solutions rapidly and fully homogenized the sputum, thus facilitating disinfection and subsequent washing of the containers. Under practical conditions, steeping for 1 hour in a 1% activated solution of chloramine, at 18 to 20° C. with 5 litres of the solution per kg. of infected linen, ensured its complete disinfection. Under the same conditions, a complete disinfection of linen was effected with a 3% non-activated solution in 3 to 4 hours ; it is recommended that handkerchiefs should be treated separately, the time of exposure being double that of the infected linen. *H. P. Fox.*

BIOCHEMISTRY

Clinical Intoxication with Potassium: Its Occurrence in Severe Renal Insufficiency. KEITH, N. M., and BURCHELL, H. B. (1949). *Amer. J. med. Sci.*, 217, 1.

Thirteen fatal cases of hyperpotassaemia occurring during serious renal insufficiency were investigated by the authors. The potassium level in serum varied between 7.7 and 10.5 milliequivalents per litre and the condition was associated with diagnostic electrocardiographic changes but not with any typical clinical signs. A concomitant rise in blood urea and blood creatinine was present in all cases. The authors attribute the infrequent occurrence of potassium intoxication in their cases of uraemia to the treatment of these patients with large intravenous infusions containing glucose and whole blood. Other changes in serum electrolytes and in *p*H make the evaluation of the toxic effects difficult in these patients. Electrocardiographic findings are diagnostic and include T waves with a high peak and a narrow base, increased intraventricular conduction time, loss of P waves, gross intraventricular conduction defects simulating right bundle-branch block, and eventually cardiac arrest. These changes are not constantly related to serum potassium levels and may return to normal after an injection of calcium, as demonstrated in two patients. In one case electrocardiographic changes started when the serum potassium level was relatively low (7.7 mEq.) and progressed without further rise in potassium level. The electrocardiographic records of six of the patients are discussed in detail. The authors emphasize the danger of giving potassium salts to patients with severe renal disease.

Electrocardiographic Manifestations of Potassium Intoxication. STEWART, H. J., SHEPARD, E. M., and HORGER, E. L. (1948). *Amer. J. Med.*, 5, 821.

Details are given of 2 cases in which auricular standstill and widespread intraventricular block were associated with marked rises in the serum potassium level. The first was in a man, aged 72, who was admitted to hospital because of bronchopneumonia and diarrhoea. To alkalinize the urine he was given 4 g. of potassium bicarbonate three times a day. Two days later, after he had had 20 g. of potassium bicarbonate, the pulse rate was 42 per minute, the pulse totally irregular, and the blood pressure 80/48 mm. Hg. An electrocardiogram showed auricular standstill with the pacemaker arising irregularly from a focus in the right ventricle; QRS was 0.15 second. The serum potassium level was 10.3 mEq. per litre, and the serum sodium level 128 mEq. per litre. The potassium bicarbonate was immediately withdrawn, and the next day the pulse was 90 per minute and regular, and the blood pressure 118/60 mm. Hg; the electrocardiogram had returned to its original configuration, and the serum potassium level was 5.9 mEq. per litre. The patient subsequently made an uneventful recovery. The second case was in a male child aged 3½ years, in the nephrotic stage of subacute glomerulonephritis complicated by a respiratory infection. The serum potassium was 10.6 mEq. per litre and again the electrocardiogram showed auricular standstill with the pacemaker arising irregularly from a focus in the right ventricle. It is pointed out that some of the new salt substitutes used in the treatment of cardiac oedema contain relatively large amounts of potassium and should not be used if there is any evidence of renal failure. For the treatment of potassium intoxication, prompt intravenous administration of physiological saline and hypertonic glucose is recommended.

Relation of Abnormalities in Concentration of Serum Potassium to Electrocardiographic Disturbances. TARAIL, R. (1948). *Amer. J. Med.*, 5, 828.

This is a study of the electrocardiographic changes and serum potassium concentrations in 19 patients with severe renal insufficiency and in 5 patients with low serum potassium levels and normal renal function. In 4 of the patients with impaired renal function there were abnormally high serum potassium levels, and in all these electrocardiographic changes were found which were not present in the remaining cases. These changes consisted of peaked T waves and increase in duration of QRS. There was also prolongation of the P-R interval. The peaked T wave was not necessarily abnormally high. No electrocardiographic changes were found with a serum potassium level below 6.8 mEq. per litre; they were sometimes present with values between 6.8 and 7.6 mEq. per litre, and always present when the serum potassium level was above 7.8 mEq. per litre. In the patients with low serum potassium concentrations the main electrocardiographic changes were low amplitude of T and prolongation of the Q-T interval.

HAEMATOLOGY

Aminopterin (A Folic Acid Antagonist) in the Treatment of Leukemia. MEYER, L. M., FINK, H., SAWITSKY, A., ROWEN, M., and RITZ, N. D. (1949). *Amer. J. clin. Path.*, 19, 119.

In this paper are described the effects of treatment with aminopterin of 43 patients with leukaemia. In only four patients (all children with acute leukaemia) was there a definite but temporary improvement in the blood picture. In 14 patients moderate or severe leucopenia developed, associated with bone marrow hypoplasia. The authors emphasize the toxicity of aminopterin and its unpredictability. The whole report is not encouraging.

Use of a Folic Acid Antagonist in Chronic Leukemia. BERMAN, L., AXELROD, A. R., VONDER HEIDE, E. C., and SHARP, E. A. (1949). *Amer. J. clin. Path.*, 19, 127.

Nine patients with chronic leukaemia (five of the myeloid and four of the lymphatic type) were treated with aminopterin, the maximum overall dose being 132 mg. No benefit resulted although the doses were sufficiently large to produce toxic effects.

The Use of Folic Acid Antagonists in the Treatment of Acute and Subacute Leukemia. A Preliminary Statement. DAMESHEK, W. (1949). *Blood*, 4, 168.

The results are given of treatment with aminopterin and related compounds of 35 patients with acute or subacute leukaemia (31 adults). It is considered necessary to give doses large enough to cause general toxic effects. When remissions have occurred attempts have been made to continue treatment by smaller maintenance doses given orally. Amethopterin, amino-an-fol, and a-ninopterin are less toxic drugs, but less active therapeutically. A-ninopterin may, however, be slightly less toxic in effective doses than is aminopterin. Remissions occurred in nine of the 35 patients.

Some Observations on the Effect of Folic Acid Antagonists on Acute Leukemia and Other Forms of Incurable Cancer. FARBER, S. (1949). *Blood*, 4, 160.

In this important paper are summarized the results of the treatment of children with acute leukaemia by means of aminopterin and related compounds (amethopterin and amino-an-fol). Of 60 children, rather more than 50% showed temporary clinical and haematological improvement. Two children were still alive 23 and 16 months after the onset of the disease.

Sickle Cell Disease ; Studied by Measuring the Survival of Transfused Red Blood Cells. CALLENDER, S. T. E., NICKEL, J. F., MOORE, C. V., and POWELL, E. O. (1949). *J. Lab. clin. Med.*, 34, 90.

This work demonstrates that the increased haemolysis in sickle cell disease is a result of the presence of abnormal erythrocytes. Normal corpuscles survive normally after transfusion into patients with sickle cell disease, but sickle cells transfused into normal subjects are destroyed relatively rapidly. The survival of blood exhibiting the sickle cell trait was normal in four cases and only slightly reduced in the fifth.

A Rapid Diagnostic Test for Sickle Cell Anemia. ITANO, H. A., and PAULING, L. (1949). *Blood*, 4, 66.

Sickling of erythrocytes is dependent upon a reduction in oxygen tension. The authors recommend that a reducing agent, sodium dithionite, be added to blood in order to bring this about. Sickling takes place within a few seconds in positive cases.

Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of Iron-binding Capacity of Serum in Man. RATH, C. E., and FINCH, C. A. (1949). *J. clin. Invest.*, 28, 79.

In cases of iron deficiency the serum iron level was found to be low, but the total iron-binding capacity was above normal; saturation was below 10%. In cases of chronic infection both the serum iron and total iron-binding capacity were reduced and the saturation was above 10%. The results in pregnancy and in other anaemias and the effects of injections of human globulin are also discussed.

Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXIX. The Anemia of Infection. Studies on the Iron-binding Capacity of Serum. CARTWRIGHT, G. E., and WIN-TROBE, M. M. (1949). *J. clin. Invest.*, 28, 86.

The total iron-binding capacity of serum is the sum of the serum iron content and the " unsaturated iron-binding capacity." The " percentage saturation " is calculated by dividing the observed serum iron content by the total iron-binding capacity. In patients with chronic infections the serum iron and total iron-binding capacity were found to be reduced and the percentage saturation lowered. The values rose in patients recovering from infections.

In two patients intravenous injection of metal-combining globulin raised the total iron-binding capacity to normal but did not diminish the rate of disappearance of iron from the serum. Experimental work in dogs is also referred to.

Haemolytic Disease of the Newborn : Criteria of Severity. MOLLISON, P. L., and CUTBUSH, M. (1949). *Brit. med. J.*, 1, 123.

This paper contains much valuable factual information, and is based upon a study of 74 cases, mostly treated by exchange transfusion. Five infants were born dead and 19 died later. The haemoglobin level of the cord blood was found to be of prognostic significance; no infant with a value of 14 g.% or over died. Taken in conjunction with the haemoglobin level the bilirubin content of the cord plasma also afforded some indication of the severity of the disease. The strength of the direct Coombs reaction performed on the infant's corpuscles and the amount of free antibody in the infant's serum were, however, not found to be reliable criteria of severity.

MORBID ANATOMY AND HISTOLOGY

Structure of the Glomerulus of the Human Kidney. MCMANUS, J. F. A. (1948). *Amer. J. Path.*, 24, 1259.

The basement membrane of normal and diseased human glomeruli, stained by the periodic acid-Schiff technique previously described by the author, was studied. It encloses the capillary loops, and certain infrequent stromal cells which lie in the intercapillary or axial space. This axial space is prominent in diabetes mellitus, contains inflammatory cells in acute nephritis, shows vacuolation and reticulation in eclampsia, and lipid accumulation in " lipoid nephrosis." *Charles Pike.*

Early Changes in the Human Brain After Arterial Air Embolism. (Über die ersten Veränderungen des menschlichen Gehirns nach arterieller Luftembolie.) ROESSLE, R. (1948). *Virchows Arch.*, 315, 461.

The author describes several cases of air embolism, in which the patients survived for periods varying from several hours to several days. He describes in detail the histological findings in the cerebral vessels (dilatation of capillaries and penetration of air into perivascular spaces) as well as the changes seen in the cerebral tissue around the vessels involved (penetration of air into glia and pericellular spaces of ganglion cells, infarct-like and necrobiotic areas of cerebral matter, haemorrhages). He compares the lesions with those seen in experimental air embolism, and states that cases of definite air embolism with survival in which scarring might be expected as a result of haemorrhages and necrosis have not been reported. *R. Schade.*

Significance of Agonal Changes in the Human Liver. POPPER, H. (1948). *Arch. Path.*, 46, 132.

The material for this study of the agonal changes in the liver at Cook County Hospital and the North Western University Medical School, Chicago, consisted of: (1) 226 needle biopsy specimens from 156 cases and 108 specimens obtained by surgical excision at laparotomy, some of which were fairly normal. (2) Biopsy and necropsy material available from the same patient in 38 cases. In 4 of these the interval after death was less than 48 hours. (3) Necropsy material from 351 males in the armed Forces all under 45 years and all of whom had died by accident or illness within 24 hours: (a) 96 cases of instantaneous death; (b) 255 cases in which death had occurred within 24 hours. The material was fixed in a variety of fluids, mainly Carnoy solution. Paraffin sections were used.

Cytoplasmic changes due to the absence of glycogen were seen in necropsy material. Dissociation of the liver cell cords with isolation of individual liver cells may be the result of agonal or necropsy change, and so regurgitation jaundice cannot be explained by communication between the bile capillaries and the perisinusoidal spaces, which result from this dissociation. In life the perisinusoidal spaces are almost completely obliterated, but in the agonal period these spaces open up revealing reticulin connecting the walls of the sinusoids with the parenchyma cells. Consequently in cases of instantaneous death, these perisinusoidal spaces usually remain closed. This point was confirmed by animal experiments and might be of medico-legal significance.

Peter Harvey.

Pulmonary Alveolar Lining in Bronchiectasis. WATTS, C. F., and McDONALD, J. R. (1948). *Arch. Path.*, 45, 742.

From each of 50 lungs removed surgically for bronchiectasis an average of three blocks was examined histologically—a small dilated bronchus, a portion of subpleural lung parenchyma, and, where possible, a uniformly atelectatic area. In the sections two relatively distinct types of alveolar cell lining were found: (a) square-cuboidal, or columnar, cells with dark-staining nuclei and acidophilic cytoplasm, non-phagocytic and packed in an uninterrupted layer; when found (17 cases) this type of epithelium was invariably in the peri-bronchial parenchyma, and in four instances continuity with bronchial epithelium was traceable (however, use was not made in this study of serial sections); (b) rounded-cuboidal, paler cells with vacuolated cytoplasm, often phagocytic and frequently scattered or in isolation. This second type of cell lining was found in subpleural parenchyma, in thickened interalveolar septa, and in the neighbourhood of scars, that is, distal to the terminal bronchi, in 43 of the cases. *W. S. Killpack.*

Nitrogen-mustard Therapy for Hodgkin's Disease, Lymphosarcoma, the Leukemias, and Other Disorders. WINTROBE, M. M., and HUGULEY, C. M. (1948). *Cancer*, 1, 357.

The results of the treatment of 32 patients with Hodgkin's disease were good in 17, but poor in 10. Only 3 of 11 patients with lymphosarcoma responded well and all 5 patients with reticulum-cell sarcoma died. Seven out of 11 patients with chronic myeloid leukaemia and 5 out of 14 patients with chronic lymphatic leukaemia responded well.

Leukopenia occurred in 51% of the patients, the earliest sign being a fall in the lymphocyte count during the first week.

It is concluded that nitrogen mustard therapy is most useful in Hodgkin's disease with generalized involvement and constitutional symptoms, but that nitrogen mustard usually fails in cases which have become refractory to x-ray therapy. *E. Neumark.*

Basalioma of the Rectum. (Das Basaliom des Rectums.) STELZNER, F. (1948). *Arch. klin. Chir.*, 261, 87.

The author describes 2 cases of relatively benign tumours with a structure similar to that of basal-cell carcinomata of the skin. In 1 case, that of a man of 64, there was a circumscribed nodular mass 2.5 cm. in diameter in the wall of the anal canal. In the other, a woman of 45, there was a large encircling growth causing stenosis of the canal and lower part of the rectum, with nodular extensions beneath the perianal skin. (The author refers to the paper on the same subject by Lawrence and Knowles, *Arch. Surg.; Chicago*, 1941, 43, 88.)

R. A. Willis.

Solid Teratoma of the Ovary, with Report of Five Cases. NOVAK, E. R. (1948). *Amer. J. Obstet. Gynec.*, 56, 300.

The clinical and pathological data are given of 4 cases in which there was a solid teratoma of the ovary which was malignant; the cases ended fatally. In a fifth case there was a dermoid cyst containing a large solid area which was benign. It is proposed that the terminology should be simplified as follows : (a) solid teratoma, which is considered to be malignant by definition, and (b) simple dermoid cyst. Dermoids may have a malignant phase. *Magnus Haines.*

The Organization of the Malignant Cell. I. Protoplasmic Disorganization in Tumour Cells. II. Relation of Some Physical, Morphological, and Biochemical Characteristics of Tumour Cells to the Disorganization of Protoplasm. POWELL, A. K. (1948). *J. R. micr. Soc.*, 66, 35.

The anaplasia characteristic of tumour cells is thought to be brought about by persistent disorganization of living protoplasm, especially by the incoordination of its ultrastructural protein fibrils. Many distinctive characteristics of tumour cells are explicable on the basis of this hypothesis, even though they were not used in its formulation.

[This important contribution provides a new view of neoplastic growth and should be read by all those interested.] *G. M. Findlay.*

Attempts at Cytological Differentiation of Atypical Epithelium of the Vaginal Portion of the Cervix by Tissue Culture and Phase-contrast Microscopy. (Ueber Versuche zur cytologischen Differenzierung des atypischen Portioepithels mit Hilfe von Gewebezuchtung und Phasenkontrastmikroskopie.) GLATTHAAR, E. (1948). *Schweiz. med. Wschr.*, 78, 720.

The author describes a study of the cytology of precancerous and cancerous cervical epithelium by phase-contrast microscopy of tissue cultures. In cultures from doubtfully precancerous abnormal epithelium no pathological cells appear, but cultures of genuinely precancerous lesions produce atypical cells resembling those in cultures from established carcinomata. The quantity of atypical cells in cultures is roughly proportional to the degree of anaplasia observed in histological preparations. Cultures from superficial and invasive carcinomata are indistinguishable. The cancerous transformation of the epithelium is evidently already established and irreversible before invasion begins. *R. A. Willis.*

The Practical Application of Phase-contrast to the Biologist's Microscope. TAYLOR, E. W. (1948). *J. R. micr. Soc.*, 66, 1.

The development of the phase-contrast microscope is reviewed from its first applications by Zernike (1935). The theory and methods of using the microscope are succinctly described.

[All those who use the microscope, whether for work or pleasure, should read this communication, which is as near as possible to an account of phase-contrast microscopy without tears. The paper is illustrated by magnificent photomicrographs.] *G. M. Findlay.*

REVIEWS

Photoelectric Methods in Clinical Biochemistry. By G. E. Delory. A Hilger Publication, Glasgow University Press. Pp. 90. Price 15s.

This is an excellent primer for any person contemplating the routine use of photoelectric techniques in a clinical laboratory and intending to use one of the Hilger instruments. The first three chapters are devoted to a clear and simple exposition of theory and contain some excellent data on the transmission of the commonly available Ilford and Chance filters. The next two chapters deal succinctly with the design and usage of the " Spekker " and the " Biochem " absorptiometers. The remaining chapter is devoted to working instructions of a range of methods suitable for use with either instrument, the majority of them based on the well-tried methods of Professor King and his collaborators. One wishes that space had been found to describe the design and use of the nepholometric attachment that Hilger manufacture, and it is to be hoped that a second edition may describe the " Uvispek " and its range of use in the same clear terms.

NICHOLAS MARTIN.

Scandinavian Journal of Clinical and Laboratory Investigation

The first number of a new Scandinavian journal, *The Scandinavian Journal of Clinical and Laboratory Investigation,* has been received. It is edited by a board and managing editor, Dr. Svein L. Sveinsson, for the Scandinavian Society for Clinical Chemistry and Clinical Pathology, and will appear quarterly, four issues forming a volume. Subscription, Kr.35.00.

Inquiries should be addressed to the publisher, MEDISINSK FYSIOLOGISK FORENINGS FORLAG, MEDICAL DEPT. A, RIKSHOSPITALET, OSLO, NORWAY.

The journal is excellently produced and has many line and half-tone illustrations. It is written in English.

Correction.—An error occurred on page 212 of the August issue where the legend to Fig. 1 stated that the strain used was 577/7. This should be numbered 577/5. The strains in Figs. 1 and 2 were identical.

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INDEX TO ABSTRACTS

AUTHORS OF ARTICLES ABSTRACTED